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(54) Title: **TEMPLATE-DEPENDENT LIGATION WITH PNA-DNA CHIMERIC PROBES**

(57) Abstract: The invention provides methods, kits, and compositions for ligation of PNA-DNA chimeric probes and oligonucleotides when they are hybridized adjacently to template nucleic acids using ligases and ligation reagents. Structural requirements of the chimeras for ligation include 5 to 15 contiguous PNA monomer units, 2 or more contiguous nucleotides, and a 3' hydroxyl or 5' hydroxyl terminus. The chimera and/or oligonucleotide may be labelled with fluorescent dyes or other labels. The methods include, for example, oligonucleotide-ligation assays (OLA) and single nucleotide polymorphism detection.

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TEMPLATE-DEPENDENT LIGATION WITH PNA-DNA CHIMERIC PROBES

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I. FIELD OF THE INVENTION

The invention relates generally to the fields of enzymology and nucleic acid analogs. Specifically, this invention is directed to template-dependent ligation of PNA-DNA chimeras and oligonucleotides with ligase enzymes.

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III. BACKGROUND

The covalent joining of nucleic acid probes by ligase enzymes is one of the most useful tools available to molecular biologists. When two probes are annealed to a template nucleic acid where the two probes are adjacent and without intervening gaps, a phosphodiester bond can be formed by a ligase enzyme (Whiteley, 1989). The ligation bond is formed between a 5' terminus of one probe and the 3' terminus of the other probe.

The events of annealing and ligation each require a high level of fidelity, i.e. complementarity, between the sequences of the ligating probes and the template nucleic acid. Both events are inefficient when base-pairing mismatches occur. Generally, DNA ligase can join two adjacent probes only when they perfectly complement a denatured template nucleic acid, such as a PCR product (Landegren, 1988; Nickerson, 1990). Even a single nucleotide mismatch at, or near, the ligation site of the probes will prevent ligation of the annealed probes.

Oligonucleotide ligation assays detect the presence of specific sequences in target DNA sample. For example, allelic discrimination assays rely on probes representing the complementary sequences of the allelic forms to the target. Ligation to a common, second target-complementary probe indicates the presence of the polymorphic site (Whiteley, 1989;

Landegren, 1988). Absence of ligation indicates the lack of the polymorphic site. Ligation can be detected through detectable labels on the allelic probe and electrophoretic separation of the ligation products (Grossman, 1994).

It is desirable to provide optimized probes and methods of annealing and ligation.

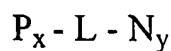
- 5 Such methods would improve assays and tests that benefit from greater precision and accuracy.

IV. SUMMARY

The invention relates to chimeric molecules of PNA and DNA monomer units and their use in ligation methods to generate ligation products. The invention is based in part on
10 the discovery that a ligase enzyme can ligate a PNA-DNA chimeric probe and a second probe under a broad range of experimental conditions and variables. PNA-DNA chimeras of the invention comprise at least two moieties covalently linked together, preferably: i) a contiguous moiety of 3 to 15 PNA monomer units, and ii) a contiguous moiety of at least two nucleotides. The nucleotide moiety has a ligatable terminus, such that the PNA-DNA
15 chimera can be ligated to a second probe.

In a first aspect, the invention provides a method of producing a template-dependent ligation product by ligating a PNA-DNA chimeric probe, annealed to a template nucleic acid in the presence of a ligase and a ligation reagent, to a second probe annealed adjacent to the chimeric probe on the template nucleic acid. The second probe is capable of
20 supporting template-dependent ligation. The second probe is a PNA-DNA chimera or an oligonucleotide. The second probe may be 5 to 100 monomer units or nucleotides (nt) in length. Preferably the second probe is 10 to 30 nt. Together, the chimeric probe and the second probe may be 10 to 100 nt.

In one illustrative embodiment of the invention, the PNA-DNA chimera has the
25 formula:



where each P is independently a PNA monomer, x is an integer from 3 to 15, L represents a covalent linkage between P and N, each N is independently a nucleotide, y is an integer from 2 to 15, and the terminal N has either a 3' hydroxyl group or 5' hydroxyl group.

30 In a preferred embodiment, the PNA moiety, i.e., P_x , of the PNA-DNA chimera is a 2-aminoethylglycine peptide nucleic acid.

The DNA moiety, i.e., N_y , of the PNA-DNA chimera may be comprised of 2'-deoxynucleotides (DNA), ribonucleotides (RNA), and modified sugars or internucleotide linkages thereof, especially those that confer greater specificity, affinity, rate of hybridization, and chemical stability.

5 The chimera and/or the second probe may be labelled with a non-radioisotopic label such that the ligation product is non-radioisotopically labelled. In embodiments employing a labelled PNA-DNA chimera, the PNA-DNA chimera may be labelled at: (i) a nucleobase, e.g. the 7-deaza or C-8 positions of a purine or a deazapurine nucleobase, or the C-5 position of a pyrimidine nucleobase; (ii) a sugar; (iii) the PNA backbone; or (iv) an amino, a sulfide,
10 a hydroxyl, and/or a carboxyl group. Preferably, the chimera is labelled at the amino terminus of the PNA moiety. In embodiments employing a labelled oligonucleotide, the oligonucleotide is preferably labelled at the opposite terminus from the ligation site, 3' or 5'. Alternatively, the oligonucleotide may be labelled at a nucleobase, but may also be labelled at other positions provided that the label does not interfere adversely with hybridization
15 affinity or specificity, or with ligase efficiency. Labels may be fluorescent dyes, fluorescence quenchers, hybridization-stabilizers, energy-transfer dye pairs, electrophoretic mobility modifiers, chemiluminescent dyes, amino acids, proteins, peptides, enzymes, and affinity ligands. Preferably, the label is detectable upon illumination with light, e.g. laser sources at infrared, visible or ultraviolet excitation wavelengths.

20 The PNA and DNA moieties of the chimeric probe are covalently linked together. The linkage, L, between the PNA and DNA moieties may be a bond, e.g. the carbonyl-nitrogen bond in an amide group where the moieties are linked without intervening atoms, or a multi-atom linker. The linkage may comprise a phosphodiester group or a phosphoramidate group.

25 The template or target nucleic acid can be any nucleic acid or nucleic acid analog capable of mediating template-directed nucleic acid synthesis. Examples of suitable template nucleic acids include, e.g., genomic DNA, DNA digests, DNA fragments, DNA transcripts, plasmids, vectors, viral DNA, PCR products, RNA, and synthetic nucleic acids. The template nucleic acid may also be a metaphase or interphase chromosome. Preferably,
30 the chromosome is denatured prior to PNA-DNA chimera hybridization and ligation. Template nucleic acids may be single-stranded or double-stranded and can range from as few as about 20-30 to as many as millions of nucleotides (nt) or base-pairs (bp), depending on the particular application.

The template nucleic acid, the PNA-DNA chimera, or the second probe may be immobilized on a solid substrate. Ligations may be conducted where one of the probes or template is attached to a solid support or surface.

V. BRIEF DESCRIPTION OF THE DRAWINGS

- 5 FIG. 1 Structures of PNA and PNA-DNA chimeras with: (1A) two 2'-deoxynucleotides, and (1B) three 2'-deoxynucleotides. B is a nucleobase.
- FIG. 2 Structures of linker reagents and linkages: (2A) linker reagents to form amide and phosphodiester linkages, (2B) bis-amide linkage of 2-(2-aminoethoxy)ethoxyacetic acid, and (2C) amide, phosphate linkage of 2-(2-aminoethoxy)ethanol.
- 10 FIG. 3 Generalized schematic of ligation: (3A) between a 3'-hydroxyl PNA-DNA chimera and a 5'-phosphate oligonucleotide hybridized to a DNA template with DNA ligase to form a PNA-DNA ligation product, and (3B) probe sequences and a 38nt perfect match DNA template for ligation experiments.
- FIG. 4 Scanned images of PAGE analysis of ligation experiments: (4A top) with T4 DNA ligase and (4A bottom) without ligase; and (4B) quantitative estimate of ligation by densitometry, SpotDenso program.
- 15 FIG. 5 Scanned images of PAGE analysis of ligation experiments with T4 DNA ligase: (5A) SYBR-Green stained gel image; and (5B) schematic of ligation of PNA, PNA-DNA chimera, and DNA to 5'-phosphate oligonucleotides hybridized to a DNA template 38nt.
- 20 FIG. 6 Scanned image of PAGE analysis of PNA-DNA chimera ligase reactions detecting wild-type and mutant sequences. Specificity of PNA-DNA chimeric probe: oligonucleotide ligation relative to oligonucleotide:oligonucleotide ligation.
- FIG. 7 MALDI-TOF Mass Spectroscopy analysis of ligation reaction products: (7A) without ligase and (7B) with ligase.
- 25 FIG. 8 Oligonucleotide Ligation Assay (OLA) with PNA-DNA chimeric probes. (8A) Determining the nature of a locus with different dye labels; (8B) Multiplex OLA with mismatched base at 5'-phosphate of oligonucleotides of different lengths and/or mobility modifiers; (8C) Multiplex OLA with mismatched base at 3' terminus of PNA-DNA chimeras of different lengths and/or mobility modifiers
- 30 FIG. 9 Oligonucleotide ligation assay with PNA-DNA chimera probes to discriminate mutations in human CFTR loci: (9A) human pCFTR621G-T: exon 4; (9B) human pCFTR1078delT: exon 7; (9C) human pCFTRG551D: exon 11.

(UPPER CASE - PNA, lower case – DNA); (9D) OLA with PNA_{10mer}-DNA_{3mer} at CFTR locus 621G-T. Visualized and recorded under UV illumination (top) and with SYBR Green staining (bottom).

5 VI. DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Reference will now be made in detail to the preferred embodiments of the invention. While the invention will be described in conjunction with the preferred embodiments, it will be understood that they are not intended to limit the invention to those embodiments. On the contrary, the invention is intended to cover alternatives,
10 modifications, and equivalents, which may be included within the invention as defined by the appended claims.

VI.1 DEFINITIONS

Unless stated otherwise, the following terms and phrases as used herein are intended to have the following meanings:

15 "Nucleobase" refers to a nitrogen-containing heterocyclic moiety, e.g. a purine, a 7-deazapurine, or a pyrimidine. Typical nucleobases are adenine, guanine, cytosine, uracil, thymine, 7-deazaadenine, 7-deazaguanine, and the like.

"Nucleoside" refers to a compound consisting of a nucleobase linked to the C-1' carbon of a ribose sugar.

20 "Nucleotide" refers to a phosphate ester of a nucleoside, as a monomer unit or within a nucleic acid. Nucleotides are sometimes denoted as "NTP", or "dNTP" and "ddNTP" to particularly point out the structural features of the ribose sugar. "Nucleotide 5'-triphosphate" refers to a nucleotide with a triphosphate ester group at the 5' position. The triphosphate ester group may include sulfur substitutions for the various oxygens, e.g. α -thio-nucleotide
25 5'-triphosphates.

As used herein, the term "nucleic acid" encompasses the terms "oligonucleotide" and "polynucleotide" and means single-stranded and double-stranded polymers of nucleotide monomers, including 2'-deoxyribonucleotides (DNA) and ribonucleotides (RNA). The nucleic acid may be composed entirely of deoxyribonucleotides, entirely of ribonucleotides,
30 or chimeric mixtures thereof, linked by internucleotide phosphodiester bond linkages, and associated counterions, e.g., H^+ , NH_4^+ , trialkylammonium, Mg^{2+} , Na^+ and the like. Nucleic acids typically range in size from a few monomeric units, e.g. 5-40 when they are commonly referred to as oligonucleotides, to several thousands of monomeric units. Unless denoted

otherwise, whenever an oligonucleotide sequence is represented, it will be understood that the nucleotides are in 5' to 3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, unless otherwise noted.

- 5 The term "Watson/Crick base-pairing" refers to the hydrogen-bonding base pairing commonly observed in double-stranded DNA.

"Attachment site" refers to a site on a moiety, e.g.: a chimera or nucleotide, to which is covalently attached a linker.

- 10 "Linker" refers to a moiety that links one moiety to another, e.g.: (i) a label to an oligonucleotide or PNA-DNA chimera, or (ii) the PNA moiety to a DNA moiety in a PNA-DNA chimera.

"PNA-DNA Chimera" refers to an oligomer comprised of: (i) a contiguous moiety of PNA monomer units and (ii) a contiguous moiety of nucleotide monomer units with an enzymatically-extendable terminus.

- 15 "Alkyl" refers to a saturated or unsaturated, branched, straight-chain, branched, or cyclic hydrocarbon radical derived by the removal of one hydrogen atom from a single carbon atom of a parent alkane, alkene, or alkyne. Typical alkyl groups include, but are not limited to, methyl, ethyl, propyl, butyl, and the like. In preferred embodiments, the alkyl groups consist of 1-12 saturated and/or unsaturated carbons.

- 20 "Cycloalkyl" refers to a cyclic alkyl radical. Nitrogen atoms with cycloalkyl substituents may form aziridinyl, azetidiny, pyrrolidinyl, piperidinyl, larger rings, and substituted forms of heterocycles thereof.

- 25 "Alkyldiyl" refers to a saturated or unsaturated, branched, straight chain or cyclic hydrocarbon radical of 1-20 carbon atoms, and having two monovalent radical centers derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent alkane, alkene or alkyne. Typical alkyldiyl radicals include, but are not limited to, 1,2-ethyldiyl, 1,3-propyldiyl, 1,4-butyldiyl, and the like.

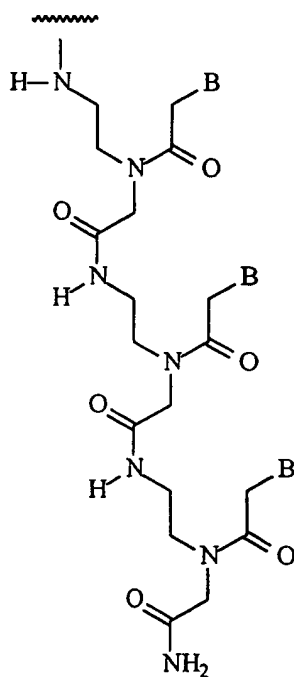
- 30 "Aryldiyl" refers to an unsaturated cyclic or polycyclic hydrocarbon radical of 6-20 carbon atoms having a conjugated resonance electron system and at least two monovalent radical centers derived by the removal of two hydrogen atoms from two different carbon atoms of a parent aryl compound. Typical aryldiyl groups include, but are not limited to, radicals derived from benzene, substituted benzene, naphthalene, anthracene, biphenyl, and the like.

"Label" refers to any non-radioisotopic moiety covalently attached to a chimera or nucleotide that is detectable or imparts a desired functionality or property in the ligation extension product.

"Ligation" is the enzymatic joining by formation of a phosphodiester bond between a PNA-DNA chimeric probe and a second probe oligonucleotide when the chimera and the second probe are hybridized (annealed) adjacently and to a template nucleic acid.

VI.2 PNA-DNA CHIMERA

In one aspect, the present invention utilizes chimeric probes which contain PNA moieties and DNA moieties. The PNA moieties may be any backbone of acyclic, achiral, and neutral polyamide linkages to which nucleobases are attached. A preferred form of the PNA moiety is a backbone of N-(2-aminoethyl)-glycine, a peptide-like, amide-linked unit (Buchardt, 1992; Nielsen, 1991), as shown below in a partial structure with a carboxyl-terminal amide:



PNA oligomers themselves are not substrates for nucleic acid processing enzymes, such as DNA polymerases (Lutz, 1999; Kyger, 1998; Lutz, 1997).

PNA-DNA chimeras are oligomers comprised of: 1) a contiguous moiety of PNA monomer units and 2) a contiguous moiety of nucleotides. The two moieties are covalently linked together. The nucleotide moiety of the chimera may be 2'-deoxynucleotides, ribonucleotides, or a mixture thereof. The nucleotide moiety of the chimera has a 3' hydroxyl terminus. The preferred length of the PNA moiety is from 3 to 15 PNA monomer

units, reflecting optimum enzymatic activity, hybridization specificity and affinity, economy of synthesis reagents, and ease of chimera synthesis and purification. The length of the DNA moiety is from 2 to 15 nucleotides. The preferred length of the DNA moiety is the shortest sequence which promotes efficient ligation, i.e. at least two 2'-deoxynucleotides (Figure 1A).

Preferred nucleobases in one or more PNA monomer units include, but are not limited to, adenine, guanine, cytosine, uracil, thymine, 7-deazaadenine, 7-deazaguanine, C-5-alkyl pyrimidines, 2-thiopyrimidine, 2,6-diaminopurine, C-5-propyne pyrimidine, phenoxazine (Flanagan, 1999), 7-deazapurine, isocytidine, pseudo-isocytidine (Egholm, 1995), isoguanosine, 4(3 *H*)-pyrimidone, hypoxanthine, and 8-oxopurines (Meyer, 1994).

The increased affinity and specificity (Egholm, 1993; Jensen, 1997) conferred by the PNA moiety in a PNA-DNA chimera allows for shorter probes to be used in hybridization experiments and assays (Uhlmann and Peyman, 1998; Uhlmann, 1998; Cook, 1997; Uhlmann, 1996. In general, shorter probes are more specific than corresponding longer probes, i.e. the relative structural perturbation is larger in a smaller probe. Also, shorter probes are more economical, i.e. cheaper to synthesize, and require less sequence information to design. It is desirable to provide methods by which PNA-DNA chimeras can be ligated to oligonucleotides and other PNA-DNA chimeras to form PNA-containing ligation products.

Binding of the PNA moiety in a PNA-DNA chimera to its DNA or RNA complement can occur in either a parallel or anti-parallel orientation. The anti-parallel duplex, where the carboxyl terminus of PNA is aligned with the 5' terminus of the complement DNA, and the amino terminus of PNA is aligned with the 3' terminus of the DNA complement, is typically more stable than the parallel duplex, where the carboxyl terminus of PNA is aligned with the 3' terminus of the DNA complement and the amino terminus of PNA is aligned with the 5' terminus of the DNA complement (Koppitz, 1998; Egholm, 1993). The exemplary chimeras shown here are designed such that the PNA moiety anneals in the anti-parallel orientation with the target sequences. Whenever a PNA sequence is represented as a series of letters, it is understood that the amino terminus is at the left side and the carboxyl terminus is at the right side.

Chimera sequences are typically completely complementary to a portion of the target sequence. However, chimera sequences may contain mixed-base ("redundant" or "degenerate") sites whereby a chimera sample may be a mixture of sequences with one or

more base positions represented by two or more different nucleobases. The mixed-base site may be located in the PNA or DNA moieties of the oligomer. Mixed-base chimeras are mixtures of sequences with varying levels of complementarity to a particular target sequence. Mixed-base chimeras may be useful for random priming or where template
5 sequence information is unknown or uncertain.

Although certain features of the invention are illustrated herein using single-stranded probes and template nucleic acids, it will also be appreciated that any of the probes and template nucleic acids may contain double-stranded regions. It is also contemplated that PNA-DNA chimeras may undergo ligation as one or both strands of a duplex ligating with a
10 second duplex, where both strands of each duplex may ligate with overhangs ("sticky ends"). For example, the chimeric probe can be provided in double-stranded form with a sticky end such that the overhang strand contains the DNA moiety and at least a portion of the PNA moiety which is complementary to the template nucleic acid, and such that the recessed strand of the chimeric probe, upon hybridization of the chimeric probe to the
15 template, is positioned either immediately adjacent to, or spaced by a gap of one or more nucleotide positions from, a terminal end of the template nucleic acid. Also contemplated are templates containing a PNA moiety of one or more PNA monomers which allow ligation of adjacently hybridized probes.

PNA-DNA chimeras can be synthesized by covalently linking PNA monomers and
20 nucleotides in virtually any combination or sequence, using the respective conventional methods of synthesis of PNA oligomers, DNA oligonucleotides, and RNA oligonucleotides (Vinayak, 1997; Uhlmann, 1996; Van der Laan, 1997). Efficient and automated methods have been developed for synthesizing PNA/DNA chimera at a 2-25 μ mole scale on commercially available, automated synthesizers, e.g. "Expedite™", Model 433A and Model
25 394 Synthesizers (PE Biosystems), and with commercially available reagents (Uhlmann, 1996; Vinayak, 1997; Van der Laan, 1997). In this approach, the chimeras can be made continuously, in a single column and on a single synthesizer.

Typically, synthesis of chimeras is initiated by detritylation of the 5'-dimethoxytrityl (DMT) group of commercially available, high-cross link, non-swelling polystyrene beads
30 packed in a synthesis column. The supports are loaded at 20-30 μ mole/g with 5'-DMT deoxynucleosides (A^{bz} , G^{bu} , C^{bz} , T) linked through the 3' hydroxyl to the support through a base-labile succinate/hydroxymethylbenzoic acid linker (Vinayak, 1997). 5'-DMT, 3'-cyanoethyl phosphoramidite deoxynucleoside monomers (Beaucage, 1992) are dissolved in

dry acetonitrile and delivered concurrently with tetrazole activator and coupled to the support-bound 5'-hydroxyl. Coupling is followed by capping with acetic anhydride of unreacted 5'-hydroxyls, and iodine oxidation to the pentavalent internucleotide phosphate triester. The DNA synthesis cycle is repeated until the last deoxynucleoside addition, where
 5 a 5' monomethoxytrityl (MMT) amino nucleoside phosphoramidite is employed to furnish a 5' amino terminus on the support-bound DNA moiety, for coupling to a PNA monomer at the linkage between DNA and PNA in the chimera. The MMT group is favored for protection of the backbone amino in the synthesis of PNA-DNA chimeras because of its acid-lability. The MMT group is efficiently and rapidly removed from nitrogen under mild
 10 acidic conditions which do not cause depurination or other damage to the chimera.

To initiate synthesis of the PNA moiety, the 5' MMT group is removed with 3% trichloroacetic acid in dichloromethane and the amino group is coupled with a PNA monomer and a coupling reagent. The backbone amino group of the PNA monomers is preferably protected with MMT and the nucleobase exocyclic amines are protected as A^{bz},
 15 G^{ibu}, and C^{bz} (Breipohl, 1997; Finn, 1996; Will, 1995). Any conventional peptide coupling reagent may be used, but HBTU and HATU are preferred coupling reagents. PNA monomers may be dissolved in 1:1 DMF:acetonitrile to a concentration of about 0.2M. Prior to delivery to the synthesis column, the monomer solution is mixed with an equal volume of 0.2M HBTU (O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium
 20 hexafluorophosphate), also in 1:1 DMF:acetonitrile (Vinayak, 1997). The solution is delivered to the column concurrently with 0.2M diisopropylethylamine in 1:1 DMF:acetonitrile. The synthesis cycles for the PNA and DNA moieties in a chimera on a Model 394 synthesizer at a 2 µmole scale are summarized in Table 1 below.

Table 1 PNA-DNA chimera synthesis cycles

Step	Function	Reagents	PNA	DNA
			Time (sec)	Time (sec)
1	Detritylation	3% CCl ₃ CO ₂ H in CH ₂ Cl ₂	60	25
2	Coupling	PNA: 0.2 M PNA monomer, HBTU, DiPEA in 1:1 DMF:CH ₃ CN DNA: 0.1 M DNA monomer,	960	25

		0.5 M tetrazole in CH ₃ CN		
3	Capping	Ac ₂ O, lutidine, N-methylimidazole, THF	25	15
4	Oxidation	iodine, pyridine, H ₂ O, THF	not required	25

After synthesis is complete, the amino terminus may be acetylated to minimize migration or cyclization, or reacted as a nucleophile in labelling. The crude chimera is cleaved from the support, and all protecting groups are removed with concentrated ammonium hydroxide at 55 °C for 8-16 hours. The chimeras are analyzed and purified by reverse-phase HPLC or polyacrylamide gel electrophoresis (PAGE), analyzed by mass spectroscopy, and quantified by correlating UV absorbance at 260 nm with mass.

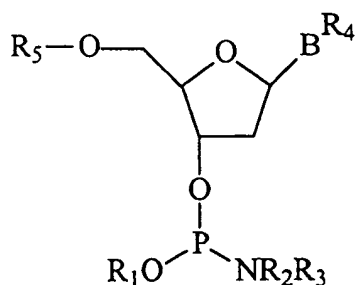
Chimeras with a DNA moiety comprising ribonucleotides can be synthesized with the appropriate RNA phosphoramidite nucleosides and/or 5' DMT protected ribonucleotides support (Vinayak, 1994). The 2' hydroxyl of RNA phosphoramidites are typically protected with the tert-butyldimethylsilyl (TBDMS) group and the exocyclic amino groups of the nucleobases are protected as A^{bz}, G^{dmf}, C^{bz}. After synthesis, TBDMS groups are removed with a fluoride reagent, e.g. tetrabutylammonium fluoride in tetrahydrofuran. Otherwise, the synthesis, purification, and analysis methods for ribonucleotide-containing PNA-DNA chimeras are virtually the same as for chimeras with only 2'-deoxynucleotide containing DNA moieties.

The linkage between the PNA and DNA moieties of the chimeric probes of the invention may be a direct bond, e.g. an amide bond formed by the amino group at the 5' of a deoxynucleotide and the carboxyl group at the carboxyl terminal of the PNA moiety without an intervening atom (Figures 1A-1B). Alternatively, the linkage L may be a phosphodiester or phosphoramidate group. The linkage may also comprise one or more units of a non-base pairing moiety such as ethyleneoxy, linked to the PNA and DNA moieties by amide (Figure 2B) or phosphate (Figure 2C) bonds. Ethyleneoxy linkage units between the PNA and DNA moieties can be installed by coupling reagents such as protected forms of 2-[2-(2-aminoethoxy) ethoxy] acetic acid. The O-linker, 2-[2-(2-aminoethoxy) acetic acid, is coupled as the MMT-amino protected amide-forming carboxylic acid, or phosphoramidite synthons (Figure 2A). One or more O linker units can act as a flexible, non-base pairing, linkage between the PNA and DNA moieties. Figure 2 shows a bis-

ethyleneoxy-acetamido linker (2B) and a bis-ethyleneoxy-phosphate linker (2C). Other exemplary linkers include alkyldiyl, e.g. hexyldiyl (Vinayak, 1997), or 1,4-phenyldiyl (Figure 2A).

VI.3 OLIGONUCLEOTIDES

5 Generally, the oligonucleotides of the present invention are prepared by the phosphoramidite synthesis method, preferred because of its efficient and rapid coupling and the stability of the starting nucleoside monomers (Caruthers, 1983; Beaucage, 1983; Beaucage, 1992). The phosphoramidite method entails cyclical addition of nucleotide monomer units to an oligonucleotide chain growing on a solid-support, most commonly in
10 the 3' to 5' direction in which the 3' terminus nucleoside is attached to the solid-support at the beginning of synthesis. The method is usually practiced using automated, commercially available synthesizers (Caruthers, 1984). Typically, phosphoramidite nucleoside monomer units include:



15 where, R_1 is a protecting group or substituent, e.g. cyanoethyl, methyl, lower alkyl, substituted alkyl, phenyl, aryl, and substituted aryl; R_2 and R_3 are amine substituents, e.g. isopropyl, morpholino, methyl, ethyl, lower alkyl, cycloalkyl, and aryl; R_4 is an exocyclic nitrogen protecting group such as benzoyl, isobutyryl, acetyl, phenoxyacetyl, aryloxyacetyl, dimethylformamidine, dialkylformamidine, and dialkylacetamidine; and R_5 is an acid-labile
20 protecting group such as DMT, MMT, pixyl, trityl, and trialkylsilyl.

Preferred nucleobases in one or more nucleosides include, but are not limited to, adenine, guanine, cytosine, uracil, thymine, 7-deazaadenine, 7-deazaguanine, C-5-alkyl pyrimidines, 2-thiopyrimidine, 2,6-diaminopurine, C-5-propyne pyrimidine, phenoxazine (Flanagan, 1999), 7-deazapurine, isocytidine, pseudo-isocytidine (Egholm, 1995),
25 isoguanosine, 4(3 *H*)-pyrimidone, hypoxanthine, and 8-oxopurines (Meyer, 1994).

Preferred sugars in one or more of the nucleosides include, but are not limited to, 2'-deoxyribose, ribose, and 2'- or 3'-ribose modifications where the 2'- or 3'-position may be hydrogen, hydroxy, methoxy, ethoxy, allyloxy, isopropoxy, butoxy, isobutoxy, methoxyethyl,

alkoxy, phenoxy, azido, amino, alkylamino, fluoro, chloro and bromo.

Other preferred sugars include 4'- α -anomeric nucleotides, 1'- α -anomeric nucleotides, and 2'-4'-linked and other "locked", bicyclic sugar modifications (Wengel, 1999).

5 VI.4 LIGASE ENZYMES

The ligase enzyme used in the present invention can be any ligase that performs ligation of the PNA-DNA chimeric probe to the second probe, when the chimeric probe and second probe are annealed to adjacent regions in a target template. DNA ligases join DNA sequences by forming a phosphodiester bond between a 5'-phosphate and a 3'-hydroxyl on
10 two probes which are adjacent, i.e. hybridized immediately next to each other (Kornberg, 1980; Whiteley, 1989). Alternatively, the 3'-phosphate end of one probe and the 5'-hydroxyl of the other probe may form a phosphodiester bond. In the present invention, one or both probes contain a PNA moiety.

For example, the DNA ligase from bacteriophage T4 can join both DNA and RNA
15 sequences, and it can use either DNA or RNA templates to align the sequences to be ligated. Reactions involving only DNA strands proceed with greater efficiency. Two DNA duplexes with base-paired blunt ends can be joined by the phage ligase. Certain ligases need cofactors such as NAD or ATP.

A number of ligases have recently been isolated from thermophile organisms and
20 which have significant activity above 60 °C and survive conditions that denature DNA. A preferred thermostable ligase is derived from *Thermus aquaticus* (Takahashi, 1984) and can also be prepared recombinantly (Barany, WO 91/17239, 1991). The thermostable ligases also exhibit a reduced activity of joining duplex DNA with blunt ends or short complementary overhang ends. These properties result in increased specificity of detection
25 and convenience in many analytical assays and applications involving ligation.

VI.5 LABELS

It is desirable to provide methods by which labelled PNA-DNA chimeras and labelled oligonucleotides can be enzymatically ligated as probes to form non-radioisotopically labelled ligation products. Fluorescence has largely supplanted
30 radioactivity as the preferred detection method for many ligation experiments and applications, such as the oligonucleotide ligation assay and other *in vitro* DNA probe-based diagnostic tests. Therefore, fluorescent labels are a preferred class of detection labels.

Labels which enhance hybridization specificity and affinity are also preferred, e.g. minor-groove binders. Affinity ligand labels are also preferred. Biotin is a useful affinity ligand label for chimeric probes and oligonucleotides for capture and isolation of ligation products. In certain experiments, biotin labelling of the template nucleic acid may be useful for

5 capture, isolation, removal, or retrieval purposes.

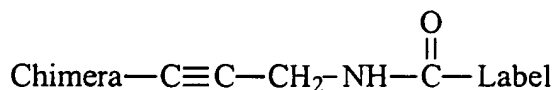
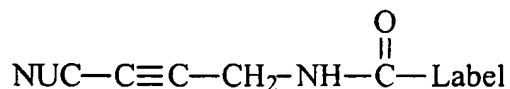
The PNA-DNA chimeras and the oligonucleotides participating in ligation may bear covalently attached labels. Labeling can be accomplished using any one of a large number of known techniques employing known labels, linkages, linking groups, standard reagents and reaction conditions, and analysis and purification methods. Generally, the linkage linking the

10 dye and oligonucleotide or chimera should not (i) interfere with ligation, (ii) inhibit ligase activity, or (iii) adversely affect the fluorescence properties of the dye, e.g. quenching or bleaching.

PNA-DNA chimeras and oligonucleotides can be labelled at sites including a nucleobase, a sugar, the aminoethylglycine backbone, amino, sulfide, hydroxyl, and

15 carboxyl. Nucleobase label sites generally include the 7-deaza or C-8 positions of the purine or deazapurine, and the C-5 position of the pyrimidine. The linkage between the label and the chimera or oligonucleotide (NUC) may be acetylenic-amido or alkenic-amido linkages (Khan, 1998). Typically, a carboxyl group on the label is activated by forming an active ester, e.g. N-hydroxysuccinimide (NHS) ester and reacted with an amino group on the alkynylamino- or

20 alkenylamino-derivatized chimera or nucleotide. The resulting linkage is 3-(carboxy)amino-1-propyn-1-yl having the structures:



Labels may be attached to oligonucleotides at any suitable terminal or internal attachment sites, including: (i) a terminus, e.g. 5' and/or 3' (Mullah, 1998), (ii) an

25 internucleotide linkage, (iii) a sugar, or (iv) a nucleobase. Labels are most conveniently and efficiently introduced at the 5' terminus with fluorescent dyes (FAM, HEX, TET) and other labels which have been functionalized as phosphoramidite reagents, as part of the automated protocol (Theisen, 1992).

A preferred class of labels provide a signal for detection of the labelled oligonucleotide by fluorescence, chemiluminescence, and electrochemical luminescence (Kricka, 1992). Fluorescent dyes useful for labelling oligonucleotides include fluoresceins (Menchen, 1993), rhodamines (Bergot, 1994), energy-transfer dyes (Lee and Spurgeon, 1998), cyanines (Kubista, 1997), and metal porphyrin complexes (Stanton, 1988).

Examples of fluorescein dyes include 6-carboxyfluorescein (6-FAM), 2',4',1,4,-tetrachlorofluorescein (TET), 2',4',5',7',1,4-hexachlorofluorescein (HEX), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyrhodamine (JOE), and aromatic-substituted xanthene dyes (Benson, 1997). The 5-carboxyl, and other regio-isomers, may also have useful detection properties.

Another preferred class of labels include fluorescence quenchers. The emission spectra of a quencher overlaps with a proximal intramolecular or intermolecular fluorescent dye such that the fluorescence of the fluorescent dye is substantially diminished, or quenched, by the phenomenon of fluorescence resonance energy transfer "FRET" (Clegg, 1992). An example of FRET in the present invention is where the PNA-DNA chimeric probe is labelled with a fluorescent dye and the second probe is labelled with a fluorescence quencher. Alternatively, the chimera may be labelled with a fluorescent quencher and the second probe is labelled with a fluorescent dye. Prior to hybridization and ligation, the fluorescent dye is substantially unquenched. After ligation, the fluorescent dye of the ligation product is substantially quenched by FRET.

Particularly preferred quenchers include but are not limited to (i) rhodamine dyes selected from the group consisting of tetramethyl-6-carboxyrhodamine (TAMRA), tetrapropano-6-carboxyrhodamine (ROX), and (ii) DABSYL, DABCYL, cyanine dyes including nitrothiazole blue (NTB), anthraquinone, malachite green, nitrothiazole, and nitroimidazole compounds and the like. Nitro-substituted forms of quenchers are especially preferred.

Energy-transfer dyes are a preferred class of chimera and oligonucleotide labels. An energy-transfer dye label includes a donor dye linked to an acceptor dye (Lee and Spurgeon, 1998). Light, e.g. from a laser, at a first wavelength is absorbed by a donor dye, e.g. FAM. The donor dye emits excitation energy absorbed by the acceptor dye. The acceptor dye fluoresces at a second, longer wavelength. The donor dye and acceptor dye moieties of an energy-transfer label may be attached by a linker linking the 4' or 5' positions of the donor dye, e.g. FAM, and a 5- or 6-carboxyl group of the acceptor dye. Other rigid and non-rigid linkers may be useful.

Metal porphyrin complexes, e.g. aluminum phthalocyanine tetrasulfonate (Stanton, 1988) and chemiluminescent compounds, e.g. 1,2-dioxetane chemiluminescent moieties (Bronstein, 1990) are also preferred classes of chimera and oligonucleotide labels.

Another preferred class of labels, referred to herein as hybridization-stabilizing moieties, include but are not limited to minor groove binders (Blackburn, 1996, p.337-46), intercalators, polycations, such as poly-lysine and spermine, and cross-linking functional groups. Hybridization-stabilizing moieties may increase the stability of base-pairing, i.e. affinity, or the rate of hybridization, exemplified by high thermal melting temperatures, T_m , of the duplex. Hybridization-stabilizing moieties may also increase the specificity of base-pairing, exemplified by large differences in T_m between perfectly complementary oligonucleotide and target sequences and where the resulting duplex contains one or more mismatches of Watson/Crick base-pairing (Blackburn, 1996, pp. 15-81). Preferred minor groove binders include Hoechst 33258 (Rajur, 1997), CDPI₁₋₃ (Kutyavin, 1996), netropsin, and distamycin. Other useful labels include electrophoretic mobility modifiers, amino acids, peptides, enzymes, and affinity ligands, e.g. biotin and digoxigenin.

Linkers between a label and the PNA/DNA chimera can be an amide bond, e.g. where the active ester form of a label is coupled with an amino group of the chimera. Also, linkers can comprise alkylidyl, arylidyl, or one or more ethyleneoxy units (Rajur, 1997).

VI.6 LIGATION

Figure 3A shows a generalized schematic of ligation between a 3'-hydroxyl PNA-DNA chimera probe and a 5'-phosphate oligonucleotide, as the second probe, hybridized to a DNA template with DNA ligase to form a PNA-DNA ligation product. The first probe has a 3'-hydroxyl terminus and the second probe has a 5'-phosphate terminus. Alternatively, the first probe has a 5'-phosphate terminus and the second probe has a 3'-hydroxyl terminus.

A ligation mixture generally includes a DNA template, a PNA-DNA chimeric probe, a second probe which is another PNA-DNA chimeric probe or an oligonucleotide, ligase, and other ligation reagents. DNA probes corresponding to the PNA-DNA chimeras may be used as a control or comparison. Under typical conditions, the probes are used at a final concentration of about 1 μ M each.

Additionally, the ligation mixture may contain reagents such as 1X T4 DNA ligase buffer [50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 25 μ g/ml bovine serum albumin], and 1,000 units of T4 DNA ligase plus 10 units of T4

polynucleotide kinase in a volume of 50 μ l. The ligation reagent may also contain ligase co-factors, e.g. NAD and ATP, polyethylene glycol, EDTA, KCl, ammonium sulfate, dithiothreitol, BSA, $MgCl_2$, Tris-HCl, glycerol, water, NaCl, mercaptoethanol, and other salts or buffers. The mixture may be incubated at 22 to 25° C for 3 hours or more. During the ligation, another addition of 10 to 20 units of T4 polynucleotide kinase may be helpful for the ligation.

Typically after incubation, ligase reaction products are heat-inactivated at approximately 80 °C for about 20 minutes and placed on ice or at 4 °C for a short period. For analysis, typically, 5 to 25 pmol of the ligation product is mixed with a final concentration of 1X loading buffer (45 mM Tris base, 45 mM boric acid, 0.4 mM EDTA, 3% Ficoll, 0.02% bromophenol blue, 0.02% xylene cyanol) and denatured at 95 °C for 10 to 20 min. The sample is loaded into a 10 to 15% denaturing PAGE gel and run in 1 \times TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 100 to 160 V, 70 °C for 25 to 60 min. The ligation product is visualized by staining the gel with SYBR-Green (Molecular Probes, Eugene, OR) in a volume of 40 to 120 ml in 1 \times TBE for 10 to 30 min. The image may be captured in a gel documentation system (e.g. ChemiImager 4000 Imaging System, Alpha Innotech Corporation, San Leandro, CA).

Figure 3B shows template and probe sequences for a ligation assay in a model system. The probe sequences include two oligonucleotide probes, 6nt and 9nt, and five PNA-DNA chimeras containing 0 to 4 DNA 2'-deoxynucleotides. The oligonucleotides and chimeras hybridize to a 38nt DNA template. The template is labelled with biotin at the 3' or 5' end to facilitate isolation and transfer.

The oligonucleotide ligation assay (OLA) is a convenient, highly-stringent method that permits distinction among known DNA sequence variants (Landegren, 1988). Multiplex analysis of highly polymorphic loci is useful for identification of individuals, e.g., for paternity testing and in forensic science, organ transplant donor-receiver matching, genetic disease diagnosis, prognosis, and pre-natal counseling, and other genetic-based testing which depend on the discrimination of single-base differences at a multiplicity of loci (Delahunty, 1996). Products of a multiplex oligonucleotide ligation assay (OLA) may be resolved electrophoretically from one another and from unligated probes under denaturing conditions with fluorescence detection (Grossman, 1994). For example, Figures 8A-8C shows different assays where two PNA-DNA chimeras, a wild-type (WT) sequence chimera and a mutant sequence chimera, bear different fluorescent dyes. Only when the

mutant sequence is present in the target sample, will the mutant sequence chimera ligate to the adjacently annealed second probe (oligo) if the mutant base pair is at the ligation site (Figure 8A).

The ligation products may be discriminated by separation based on: (i) size using electrophoresis or chromatography and/or (ii) detectable labels (Grossman, 1994). With a plurality of fluorescent dyes labelled to chimeras with sequences targetting unique target sequences, multiplexed OLA can be conducted on a single sample in a single vessel. Requirements for efficient multiplex OLA include probes that anneal and ligate in a highly specific and rapid manner. The chimeras and second probe sequences may be selected such that the mutant base, or single base polymorphism, may be at the 5'-phosphate of the second probe (Figure 8B) or the 3'- terminus of the chimera (Figure 8C).

It is contemplated that OLA experiments of the present invention may be conducted on solid substrates where the template nucleic acid, PNA-DNA chimeric probe, or the second probe may be immobilized on a solid particle or bead, or a solid porous or non-porous surface. When immobilized, the template, chimera or second probe is preferably covalently attached to the solid substrate, e.g. via a terminal monomer unit. The solid substrate may be polystyrene, controlled-pore-glass, silica gel, silica, polyacrylamide, magnetic beads, polyacrylate, hydroxyethylmethacrylate, polyamide, polyethylene, polyethyleneoxy, and copolymers and grafts of any of the above solid substrates. The configuration or format of the solid substrate may be small particles or beads of approximately 1 to 50 μm in diameter, membranes, frits, slides, plates, micromachined chips, alkanethiol-gold layers, non-porous surfaces, and polynucleotide-immobilizing media.

For example, a PNA-DNA chimeric probe is covalently attached by a linker at the amino terminus to a non-porous, inorganic surface, e.g. glass (Guo, 1994). A template nucleic acid sample is allowed to hybridized to the chimeric probe under conditions that promote hybridization. A second probe with a sequence complementary to the template is added to the hybridized duplex and the second probe hybridizes to the template adjacent to the chimeric probe. The chimeric probe and second probe are ligated together with ligase. The ligated product may be detected and/or isolated where the chimeric probe, template, or second probe bear a label or affinity ligand and an oligonucleotide ligation assay is thereby performed.

In a preferred embodiment, an array of chimeric probes are assembled on a solid substrate where a chimeric probe of known sequence occupies a defined area on a two-dimensional surface. The number of chimeric probes on any particular surface may be hundreds or even thousands, limited by the spatial requirements for synthesis, attachments, and detection (Fodor, 1995). Alternatively an array of probes may be immobilized on beads or particles contained in wells or vessels. A template, or mixture of templates, may be added for hybridization to the immobilized chimeric probes on the surface. Where sufficient sequence complementarity exists under the defined hybridization conditions, duplex formation will occur. A mixture of second probes may be added separately, or with the template sample, for hybridization. In the presence of ligase, ligation will occur only where chimeric probes and second probes are hybridized adjacently. Unhybridized probes and template samples may be removed by washing under conditions that maintain hybridization, or under denaturing conditions. Where the second probe bears a label, e.g. fluorescent dye, the ligation product is covalently immobilized on the surface and can be detected, e.g. laser-induced fluorescence. From the knowledge of the immobilized chimeric probe sequences, the presence of certain sequences in the template sample can be deduced from the location(s) of detected fluorescence on the array surface.

In a second aspect of the invention, a kit for ligation is provided. In one embodiment, for example, the kit, which is useful for practicing the method of the invention, comprises: (i) a PNA-DNA chimera having from 3 to 15 contiguous PNA monomer units, from 2 to 15 contiguous nucleotides, and a 3' hydroxyl; (ii) a second probe where the second probe is a PNA-DNA chimera or an oligonucleotide and; (iii) a ligase enzyme. The chimera and/or the oligonucleotide may be labelled with a non-radioisotopic label. In another embodiment, the kit additionally includes a template comprising a sequence complementary to the chimera or containing one or more mismatches to the chimera. In another embodiment, the kit additionally includes a polynucleotide kinase.

From the foregoing discussion, it can be seen how various features and advantages of the invention are met. The present invention provides a method for detecting selected target sequences that is highly sensitive and accurate. Selected target sequences can be detected using chimeric PNA/DNA probes containing target-specific sequences shorter than all-DNA probes used in previous oligonucleotide ligation assays. The chimeric probes thus require less target sequence information to design and can be less expensive to synthesize.

In addition, the present invention can be adapted to a wide variety of target sequences and assay formats, and can be readily automated.

VI.7 EXAMPLES

The invention is further illustrated by the following examples, which are intended to be purely exemplary of the present invention and not to limit its scope in any way.

Example 1 Labelling of PNA-DNA chimera

TAMRA and NTB labelling:

Labelling is performed with 5 mg of NHS ester of TAMRA or NTB dissolved in 100 µl DMF or NMP and 10 µl DIEA. The labelling mixture is added to the support bound PNA-DNA chimera and allowed to react for 2 to 18 hours (typically overnight). The support is washed following the labelling with DMF and subsequently DCM prior to cleavage.

CDPI labelling:

CDPI₃ is attached to the chimera by three consecutive couplings of Fmoc-CDPI (Lukhtanov, 1995) to the amino terminus of a PNA-DNA chimera to give CDPI₃-labelled PNA-DNA chimera. The CDPI monomer unit, 1,2-dihydro-(3*H*)-pyrrolo[3,2-*e*]indole-7-carboxylate, protected with Fmoc (5 mg, 0.012 mmole) is dissolved in 100 µl NMP and activated by 0.95 equivalents HATU (0.2M in DMF) and 2 equivalents DIEA (0.4M in DMF). After one hour at room temperature, the activated Fmoc-CDPI solution is added to the support bound chimera and allowed to couple for another hour at room temperature. The resin is washed following the coupling with 20 ml DMF. The Fmoc is removed by treatment of the resin support with 1:4 piperidine:DMF for 10 minutes at room temperature. This coupling and deprotection cycle is repeated two additional times for a total of 3 manual couplings to give CDPI₃-labelled PNA-DNA chimera.

25 Example 2 Ligation

Figure 4 shows ligation experiments where a ligation mixture contained a template and two probes at a final concentration of 1 µM each, 1X T4 DNA ligase buffer [50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 25 µg/ml bovine serum albumin], and 1,000 units T4 DNA ligase (New England BioLabs, Beverly, MA) in a volume of 50 µl. T4 ligase can also be purchased from Boehringer-Mannheim. Approximately 70 units of NE Biolabs T4 DNA ligase is equal to 1 Weiss unit of

Boehringer-Mannheim T4 ligase. The same reactions were also conducted without ligase. After incubation at 22.5 to 25 °C for 3 to 4 hours, the reaction mixture was heated at 80 °C for 20 min. and then stored at 4 °C. Five µl of the reaction mixture was analyzed by (15%) polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (7M urea) at 120-140 V for 20-60 min (Figure 4A). Ligation of 5'-phosphate oligonucleotides (DNA2, DNA4 or DNA5) to an oligonucleotide DNA3 or PNA-DNA chimeras were conducted on templates (DNA1 or DNA6) according to Table 2. Ligation with T4 ligase (top gel image) is evident in lanes 3,4,6,8,10 between PNA-DNA chimeras and 5'-phosphate oligonucleotides by the appearance of new bands. Ligation was effective with chimeras having 6 PNA monomers and from 2 to 4 2'-deoxynucleotide monomers. Control ligation between two oligonucleotide probes (lane 2) shows a ligation product band. Control ligation with only one probe (lanes 1,5,7,9) does not show a new band. Experiments without T4 ligase (bottom gel image) show bands for the templates and probes.

Table 2. Figures 4A-4B

Lane	Template	PNA-DNA chimera or oligonucleotide	5'-phosphate oligonucleotide
1	DNA1		DNA2
2	DNA1	DNA3	DNA2
3	DNA1	PNA ₆ -DNA ₃	DNA2
4	DNA1	PNA ₆ -DNA ₃	DNA2
5	DNA1		DNA4
6	DNA1	PNA ₆ -DNA ₂	DNA4
7	DNA1		DNA5
8	DNA1	PNA ₆ -DNA ₄	DNA5
9	DNA6		DNA5
10	DNA6	PNA ₁₁ -DNA ₄	DNA5
M	DNA oligo ladder		

DNA1

(SEQ. ID NO. 9)

Biotin-cgctcaacacatagcatggtctagaactaagcctggaa

DNA6

(SEQ. ID NO. 15)

cgctcaacacatagcatggtaaagccgggacctaactgtt

	DNA3	tagttctag	(SEQ. ID NO. 2)
	PNA ₆ -DNA ₂	TAGTTC-ta	(SEQ. ID NO. 5)
	PNA ₆ -DNA ₃	TAGTTC-tag	(SEQ. ID NO. 6)
	PNA ₆ -DNA ₄	TAGTTC-taga	(SEQ. ID NO. 7)
5	PNA ₁₁ -DNA ₄	TAGGTCCCGGC-ttta	(SEQ. ID NO. 10)
	DNA2 5'-phos	accatgctatgtgttgagcg	(SEQ. ID NO. 11)
	DNA4 5'-phos	gaccatgctatgtgttgagcg-biotin	(SEQ. ID NO. 12)
	DNA5 5'-phos	ccatgctatgtgttgagcg-biotin	(SEQ. ID NO. 8)

(UPPER CASE - PNA, lower case - DNA)

10 Figure 4B shows the quantitative estimate of the ligase reactions in Figure 4A by densitometry with the SpotDenso program. The bands enclosed by the boxes are the 5'-phosphate probes remaining after ligation. The negative control experiments in lane 1,5,7, and 9 established the levels of 5'-phosphate probe remaining when no ligation occurred. The positive control experiment in lane 2 gave the ligation efficiency between two all-DNA probes. From these quantified values, the ligation efficiencies with PNA-DNA chimeric probes can be calculated from the areas (IDV - Integrated Density Value) in the chart (right).

Figure 5 shows ligation experiments using T4 DNA ligase with PNA-DNA chimeric probes to which 1 to 4 DNA bases are attached at 3' terminus of the PNA oligomer and a second probe, a 5'-phosphate oligonucleotide of variable lengths (lanes 4-7). The lengths of the chimera and the oligonucleotide probes are chosen to form ligation products of equivalent length (Table 3). In addition, control ligations were conducted where instead of the chimera, no probe (lane 2), an all PNA probe (lane 3), an all-DNA probe, 6nt (lane 8) and an all-DNA probe, 9nt (lane 9) were used. The ligation mixture contained a 38nt DNA template and the two probes at a final concentration of 1 μ M each, 1X T4 DNA ligase buffer [50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 25 μ g/ml bovine serum albumin], and 1,000 units of T4 DNA ligase in a volume of 50 μ l. The ligation reaction mixture was incubated at 22.5 to 25°C for 3 to 4 h. After incubation, the reaction mixture was heated at 80 °C for 20 min and then stored at 4 °C. Five μ l of the reaction mixture was loaded onto a 15% polyacrylamide gel and electrophoresed at 120-140

V under denaturing conditions (7M urea, 70°C) for 20-60 min., then stained with SYBR-Green. Figure 5A shows a scanned image of the stained gel. Figure 5B is a schematic of ligation of PNA, PNA-DNA chimera, and DNA to 5'-phosphate oligonucleotides hybridized to a DNA template 38nt (SEQ. ID NO. 9).

- 5 It is evident from new bands below the template bands that PNA-DNA chimeras are ligated where the chimera has 2, 3, or 4 DNA monomers (2'-deoxynucleotides), lanes 5-7 respectively. No ligation is evident for an all-PNA probe or a chimera containing only 1 DNA monomer, lanes 3 and 4 respectively. Lane 1 is a negative control. Lanes 8 and 9 are positive controls, where 6nt and 9nt oligonucleotides are ligated to 5'-phosphate
- 10 oligonucleotides. The electrophoretic retardation of PNA in the ligation products of chimeras, lanes 5-7, is evident compared to all-DNA ligation products, lanes 8 and 9.

Table 3. Figures 5A-5B

Lane	DNA Template	Probes	
		5'-phosphate DNA	PNA-DNA chimera
2	38nt	23nt	None
3	38nt	23nt	PNA ₆
4	38nt	22nt	PNA ₆ -DNA ₁
5	38nt	21nt	PNA ₆ -DNA ₂
6	38nt	20nt	PNA ₆ -DNA ₃
7	38nt	19nt	PNA ₆ -DNA ₄
8	38nt	23nt	DNA ₆
9	38nt	20nt	DNA ₉
1	M = DNA oligonucleotide ladder		

- DNA₆ tagttc (SEQ. ID NO. 1)
- 15 DNA₉ tagttctag (SEQ. ID NO. 2)
- PNA₆ TAGTTC (SEQ. ID NO. 3)
- PNA₆-DNA₁ TAGTTC-t (SEQ. ID NO. 4)
- PNA₆-DNA₂ TAGTTC-ta (SEQ. ID NO. 5)
- PNA₆-DNA₃ TAGTTC-tag (SEQ. ID NO. 6)
- 20 PNA₆-DNA₄ TAGTTC-taga (SEQ. ID NO. 7)

5'-phosphate oligos :

- 19nt 5'-phos-ccatgctatgtggtgagcg-biotin (SEQ. ID NO. 8)
- 20nt 5'-phos-accatgctatgtggtgagcg-biotin (SEQ. ID NO. 11)
- 21nt 5'-phos-gaccatgctatgtggtgagcg-biotin (SEQ. ID NO. 12)
- 22nt 5'-Phos-agaccatgctatgtggtgagcg-biotin (SEQ. ID NO. 13)
- 5 23nt 5'-Phos-tagaccatgctatgtggtgagcg-biotin (SEQ. ID NO. 14)

DNA template 38nt : (SEQ. ID NO. 16)

cgctcaacacatagcatgggtccagaactaagcctggaa

10 (UPPER CASE - PNA, lower case - DNA)

Figure 6 shows the specificity of ligation with PNA-DNA chimeric probes. PNA-DNA chimeras and 5'-phosphate oligonucleotides were ligated on templates, as perfect matches and with mismatches. Ligation mixtures contained a template and two probes at a final concentration of 1 μ M each, 1X T4 DNA ligase buffer [50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 25 μ g/ml bovine serum albumin], and 1,000 units of T4 DNA ligase in a volume of 50 μ l. Ligation reactions were incubated at 22.5 to 25°C for 3 to 4 h. After incubation, the reaction mixture was heated at 80 °C for 20 min and then stored at 4 °C. Five μ l of the reaction mixture was used for PAGE analysis,

20 electrophoresing at 120-140 V for 20-60 min.

Table 4 Figure 6

Lane	DNA Template	Phosphorylated DNA		PNA-DNA chimera or DNA oligonucleotide
		Matched	Mismatched	
1	DNA1C	DNA2		DNA ₉
2	DNA1C	DNA2	DNA2A	DNA ₉
3	DNA1C	DNA2		PNA ₆ -DNA ₃
4	DNA1C	DNA2	DNA2A	PNA ₆ -DNA ₃
5	DNA1C	DNA3		PNA ₆ -DNA ₄ (mismatched)
6	DNA2C	DNA2A		DNA ₉ (mismatched)
7	DNA2C	DNA2A		PNA ₆ -DNA ₃ (mismatched)
8	DNA2C	DNA4		PNA ₆ -DNA ₂ (mismatched)

M DNA oligonucleotide ladder

DNA Template

DNA1C (SEQ. ID NO. 27)

Biotin-cgctcaacacatagcatggcctagaactaagcctggaa

5 DNA2C (SEQ. ID NO. 16)

Biotin-cgctcaacacatagcatggtcagaactaagcctggaa

Phosphorylated DNA

DNA2 5'-Phos-gccatgctatgtggt-Biotin (SEQ. ID NO. 17)

DNA2A 5'-Phos-accatgctatgtggt-Biotin (SEQ. ID NO. 18)

10 DNA3 5'-Phos-ccatgctatgtgttgagcg-Biotin (SEQ. ID NO. 8)

DNA4 5'-Phos-accatgctatgtgttgagcg-Biotin (SEQ. ID NO. 11)

DNADNA₉ tagttctag (SEQ. ID NO. 2)PNA-DNA Chimera15 PNA₆-DNA₂ TAGTTC-ta (SEQ. ID NO. 5)PNA₆-DNA₃ TAGTTC-tag (SEQ. ID NO. 6)PNA₆-DNA₄ TAGTTC-taga (SEQ. ID NO. 7)

(UPPER CASE - PNA, lower case - DNA)

20

The experiments in Figure 6 show that PNA-DNA chimeric probes require a high level of sequence complementarity for ligation to occur. When a mismatch occurs either in the chimeric probe or the second probe, ligation is not detectable, within the limits of the system shown. By comparison, all-DNA probes are less specific. When a mismatch occurs

25 in either all-DNA probe, some ligation is still evident. Lane 1 is a positive control ligation where two oligonucleotides are perfectly matched (W) to the template and ligate to form a ligation product migrating at the expected rate of 24nt. The experiment of lane 2 additionally has a mismatched (W+M) probe which ligates, evidenced by a new band

between the perfect match product and the template (38nt). The mismatched probe, DNA2A, has a mismatch at the ligation site, the A base at the 5' terminus of the 5'-phosphate probe. The experiment of lane 3 ligates perfectly matched PNA₆-DNA₃ chimera and DNA2 second probe, giving a new band, migrating faster than template. The experiment of lane 4 additionally has the mismatched DNA2A probe. Unlike the all-DNA experiment of lane 2, mismatched DNA2A does not ligate with the chimera, demonstrating greater specificity conferred by the PNA moiety. The experiments of lanes 5 and 7 likewise have mismatches at the 3' terminus of the chimera. The 3' terminus of the chimera in the experiment of lane 8 has a deletion and a mismatch. These experiments, lanes 5,7,8 show no ligation product. The experiment of lane 6 has a mismatch in the DNA₉ probe at the penultimate base near the 3' terminus. In this experiment, a ligation product is evident, reflecting the lower specificity of ligation of all-DNA probes.

In summary, the ligation experiments shown in Figure 6 illustrate that PNA-DNA chimeric probes when ligated to oligonucleotide probes are better able to discriminate base-pair mismatches (specificity) than ligations between two all-DNA, oligonucleotide probes, whether the mismatch occurs in the chimera probe or the oligonucleotide probe.

Example 3 MALDI-TOF analysis of ligation reaction

Mass spectra were acquired on a MALDI-TOF MS (Voyager DE, PerSeptive Biosystems, division of PE Corporation) workstation. Desalted samples are mixed 1:1 with matrix solution consisting of 50 mg/ml 3-hydroxy picolinic acid, 50 mM ammonium citrate, and 30% acetonitrile, and are spotted onto a sample plate. Time-of-flight data from 20 to 50 individual laser pulses are recorded and averaged on a transient digitizer, after which the averaged spectra are automatically converted to mass by data processing software. Figure 7 of ligation reactions. A 3' biotinylated 20nt oligonucleotide (mass 6303) and a PNA-DNA chimera (mass 2539) were hybridized to 5' biotinylated DNA template 38nt (mass 12358). Figure 7A shows MALDI-TOF Mass Spectroscopy analysis of the mixture without ligase. The analysis shows only ion peaks of the starting materials. When the ligation mixture contains ligase (Figure 7B), a ligation product is evident with the expected mass of 8823.8.

30 template: (SEQ. ID NO. 9)

Biotin-cgctcaacacatagcatggtctagaactaagcctggaa

5' -phos-accatgctatgtgttgagcg-biotin (SEQ. ID NO. 11)

(mass 6303)

Ac-TAGTTC-tag (mass 2539)

(SEQ. ID NO. 6)

ligation product :

Ac-TAGTTC-tagaccatgctatgtgttgagcg-biotin
(mass 8824)

(SEQ. ID NO. 19)

5

Ac = acetylated amino terminus

(UPPER CASE - PNA, lower case - DNA)

Example 4 Multiplex Oligonucleotide Ligation Assay for CFTR loci

10 OLA reactions for CFTR loci were multiplexed in one tube. Two differentially
labelled (i.e. FAM- or TET- at 5' site) PNA-DNA chimeras and one 5' phosphorylated
DNA oligonucleotide were used for the analysis of each mutation. The sequences of the
probes and templates are given in Fig. 8. All multiplex OLA reactions are carried in a 20 µl
reaction volume containing 20 mM Tris-HCl, pH 7.6, 25 mM potassium acetate, 10 mM
15 magnesium acetate, 10 mM DTT, 1 mM NAD, 0.1% Triton X-100, 1 to 50 nM each probe,
5 to 10 µl of pooled PCR product, and 2 to 10 units of thermostable ligase such as *Thermus*
aquaticus ligase. Linear amplification of product is achieved by 20 to 30 cycles at 94 °C for
30 sec and 30 to 50 °C for 1 to 3 min, followed by heating at 95 °C for 10 to 20 min in a
Model 9700 Thermocycler (PE Biosystems division of PE Corporation).

20 An aliquot of 2 µl of each multiplex OLA product was mixed with 2.5 µl of
deionized formamide, 0.5 µl of dextran blue loading buffer, and 0.5 µl of GENESCAN-500
TAMRA size marker. The mixture was denatured at 95 °C for 3 min and then rapidly
chilled on ice prior to loading the gel. OLA products were electrophoresed for 3.5 h at
2,500 V on a Model 373A fluorescence-scanning DNA sequencer (PE Biosystems, division
25 of PE Corporation) using an 8% acrylamide, 19:1 acrylamide:bisacrylamide, denaturing gel
containing 8.3 M urea, 89 mM Tris, 89 mM boric acid, and 2 mM EDTA. The resulting gel
data are analyzed for peak color and fragment size using the GENESCAN Fragment
Analysis Software and the Genotyper Software (PE Biosystems Division of PE
Corporation).

30 Figure 9 shows the oligonucleotide ligation assay with PNA-DNA chimera probes to
discriminate mutations in human CFTR loci. Ligation mixtures contained a template and
two probes at a final concentration of 1 µM each, 1X T4 DNA ligase buffer [50 mM Tris-

HCl, pH 7.5, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 25 µg/ml bovine serum albumin], and 1,000 units of T4 DNA ligase (New England Biolabs) plus 10 units of T4 polynucleotide kinase in a volume of 50 µl. The mixtures were incubated at 22.5 to 25°C for 3 to 4 h. After incubation the reaction mixture was heated at 80 °C for 20 min and then stored at 4°C. Five µl of the reaction mixture was used for PAGE analysis, electrophoresing at 120-140 V for 20-60 min. (9A) human pCFTR621G-T: Exon 4; (9B) human pCFTR1078delT: Exon 7; (9C) human pCFTRG551D: Exon 11. (UPPER CASE - PNA, lower case - DNA)

Figure 9D shows scanned images of OLA experiments with PNA-DNA chimeric probes and an all-DNA control probe (lane 10). Ligation of a 3'-TAMRA-labelled, 5'-phosphate oligonucleotide with PNA₁₀-DNA₃ chimera at the CFTR locus 621G-T with T4 ligase gave a fluorescent labelled product, visible without staining under UV light (9D, top gel) and by staining with SYBR-Green (9D, bottom gel). Ligation products with PNA-DNA chimeric probes are also evident from experiments in lanes 3 and 9.

Table 5 Figure 9D

Lane	DNA template	PNA-DNA chimera or oligonucleotide	5'-phosphate oligonucleotide
1	1a		
2	1a		2 (FAM)
3	1a	PNA ₁₀ -DNA ₃	2
4	1b		
5	1b		3 (TAMRA)
6	1b	PNA ₁₀ -DNA ₃	3 (TAMRA)
7	1c		
8	1c		4
9	1c	PNA ₆ -DNA ₄	4
10	1c	DNA ₉	4
M	DNA oligo ladder		

DNA Template

DNA1a (SEQ. ID NO. 20)

gtttgatttataagaaggtaatacttccttgacag

DNA1b (SEQ. ID NO. 21)

cacagataaaaacaccacaaagaaccctgagaagaagaag

DNA1c (SEQ. ID NO. 9)

Biotin-cgctcaacacatagcatggtctagaactaagcctggaa

PNA-DNA Chimera

PNA ₁₀ -DNA ₃ (lane 3)	Ac-CAAGGAAGTA-tta	(SEQ. ID NO. 22)
PNA ₁₀ -DNA ₃ (lane 6)	Ac-CTTCTCAGGG-ttc	(SEQ. ID NO. 23)
PNA ₆ -DNA ₄	TAGTTC-taga	(SEQ. ID NO. 7)

DNA

5	DNA ₉	tagttctag	(SEQ. ID NO. 2)
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5'-phosphate oligonucleotides

2(FAM)	5'-Phos-ccttcttata-FAM-3'	(SEQ. ID NO. 24)
2	5'-Phos-ccttcttata-3'	(SEQ. ID NO. 24)
3(TAMRA)	5'-Phos-tttgtggtgtttt-TAMRA-3'	(SEQ. ID NO. 25)
10 4	5'-Phos-ccatgctatgtgtt-Biotin-3'	(SEQ. ID NO. 26)

All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

- 15 Although certain embodiments have been described in detail above, those having ordinary skill in the art will clearly understand that many modifications are possible in the preferred embodiments without departing from the teachings thereof. All such modifications are intended to be encompassed within the following claims.

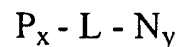
CLAIMS

We claim:

1. A method of producing a template-dependent ligation product comprising the step of enzymatically ligating a PNA-DNA chimeric probe to a second probe in the presence of a template nucleic acid and a ligase, said chimeric probe having a PNA moiety and DNA moiety, said DNA moiety having at least two nucleotides and a 3' hydroxyl or 5' hydroxyl terminus,

wherein the chimeric probe and the second probe are each hybridized to the template nucleic acid and adjacent to each other, and at least a portion of the PNA moiety is hybridized to the template, and wherein the second probe is a PNA-DNA chimera or an oligonucleotide.

2. The method of claim 1 in which the PNA-DNA chimera has the structure:



wherein:

- each P is independently a PNA monomer;
x is an integer from 3 to 15;
each N is independently a nucleotide; and
y is an integer of 2 or more;
L represents a covalent linkage between P and N;
with the proviso that the terminal nucleotide N has a 3'-hydroxyl group or a 5'-hydroxyl group.

3. The method of claim 2 wherein the 3'-terminal nucleotide N of the chimera contains a 3'-phosphate group and the 5' end of the second probe contains a 5'-hydroxyl.

4. The method of claim 2 wherein the 3'-terminal nucleotide N of the chimera contains a 3'-hydroxyl and the 5' end of the second probe contains a 5'-phosphate group.

5. The method of claim 2 wherein the 5'-terminal nucleotide N of the chimera contains a 5'-phosphate group and the 3' end of the second probe contains a 3'-hydroxyl.

6. The method of claim 2 wherein the 5' terminal nucleotide N of the chimera contains a 5'-hydroxyl and the 3' end of the second probe contains a 3'-phosphate group.
7. The method of claim 2 wherein P_x is a 2-aminoethylglycine peptide nucleic acid.
- 5 8. The method of claim 2 in which each nucleotide N is independently a 2'-deoxyribonucleotide.
9. The method of claim 2 in which each nucleotide N is independently a ribonucleotide.
- 10 10. The method of claim 2 wherein the nucleobases of N_y are selected from the group consisting of adenine, guanine, cytosine, uracil, thymine, 7-deazaadenine, 7-deazaguanine, C-5-alkyl pyrimidine, 2,6-diaminopurine, 2-thiopyrimidine, C-5-propyne pyrimidine, phenoxazine, isocytidine, pseudo-isocytidine, isoguanosine, hypoxanthine, 8-oxopurine, and 4(3 *H*)-pyrimidone.
- 15 11. The method of claim 2 wherein the sugars of N_y are each independently selected from the group consisting of 2'-O-alkyl-ribonucleotides, 2'-O-methyl-ribonucleotides, 2'-O-allyl-ribonucleotides, 2'-allyl ribonucleotides, 2'-halo-ribonucleotides, 2'-O-methoxyethyl-ribonucleotides, 4'- α -anomeric nucleotides, 1'- α -anomeric nucleotides, 2',4'-linked nucleotides, and bicyclic nucleotides.
- 20 12. The method of claim 1 in which the PNA-DNA chimera and/or the oligonucleotide are non-radioisotopically labelled.
13. The method of claim 12 wherein the PNA-DNA chimera is labelled at the amino terminus of the PNA moiety.
14. The method of claim 12 wherein the oligonucleotide is labelled at a nucleobase.
- 25 15. The method of claim 14 wherein the nucleobases are labelled at the 7-deaza or C-8 positions of the purine or deazapurine, and the C-5 position of the pyrimidine.

16. The method of claim 12 wherein the label is selected from the group consisting of fluorescent dyes, fluorescence quenchers, hybridization-stabilizers, energy-transfer dye sets, electrophoretic mobility modifiers, chemiluminescent dyes, amino acids, proteins, peptides, enzymes, and affinity ligands.
- 5 17. The method of claim 16 where the label is a fluorescent dye selected from the group consisting of FAM, TET, HEX, JOE, TAMRA, ROX, aromatic-substituted xanthene dyes, 4,7-dichloro-fluoresceins, 4,7-dichloro-rhodamines, and cyanines.
- 10 18. The method of claim 16 where the label is a fluorescence quencher selected from the group consisting of TAMRA, d-TAMRA, ROX, DABCYL, DABSYL, malachite green, NTB, and cyanines.
19. The method of claim 16 where the label is a hybridization-stabilizer that is a minor groove binder.
20. The method of claim 16 where the minor groove binder is selected from the group consisting of Hoechst 33258, CDPI₁₋₃, MGB1, netropsin, and distamycin.
- 15 21. The method of claim 16 where the label is an affinity ligand selected from the group consisting of biotin, 2,4-dinitrophenyl, digoxigenin, cholesterol, polyethyleneoxy, peptides, and fluorescein.
22. The method of claim 12 wherein the oligonucleotide is labelled at a 3' terminus.
- 20 23. The method of claim 12 wherein the oligonucleotide is labelled at a 5' terminus.
24. The method of claim 2 wherein L is selected from the group consisting of a covalent bond, phosphate, phosphoramidate, alkylidyl consisting of 1-20 carbon atoms, arylidyl consisting of 6-20 carbon atoms, O-linker, and $-(\text{CH}_2\text{CH}_2\text{O})_m-$ where m is 1 to 6.
- 25 25. The method of claim 1 in which the template nucleic acid is a DNA and the ligase is selected from the group consisting of T4 DNA ligase, *E. coli* DNA ligase, and a thermostable ligase.

26. The method of claim 1 in which the template nucleic acid is an RNA and the ligase is an RNA ligase.
27. The method of claim 1 in which the PNA-DNA chimeric probe, the second probe, or the template nucleic acid is immobilized on a solid substrate.
- 5 28. The method of claim 27 in which the PNA-DNA chimeric probe, the second probe, or the template nucleic acid is covalently attached to the solid substrate, optionally with the aid of a linker.
29. The method of claim 28 wherein the solid substrate is selected from the group consisting of polystyrene, controlled-pore-glass, glass, silica gel, silica,
10 polyacrylamide, magnetic beads, polyacrylate, hydroxyethylmethacrylate, polyamide, polyethylene, polyethyleneoxy, and copolymers and grafts of any of the above solid substrates.
30. The method of claim 28 wherein the solid substrate is selected from the group consisting of small particles, beads, membranes, frits, slides, plates, micromachined
15 chips, alkanethiol-gold layers, non-porous surfaces, and polynucleotide-immobilizing media.
31. A method of template-dependent ligation, comprising the steps of:
a) generating a ligation product by enzymatically ligating a PNA-DNA chimeric probe to a second probe in the presence of a template to which the chimeric probe and the
20 second probe are complementary and hybridized adjacently, wherein the second probe is a PNA-DNA chimera or an oligonucleotide; and
b) detecting the ligation product.
32. The method of claim 31 in which the chimeric probe and/or the second probe is non-radioisotopically labelled.
- 25 33. A kit for template-dependent ligation comprising:
a PNA-DNA chimeric probe, said probe comprising 5 to 15 contiguous PNA monomer units, 2 to 15 contiguous nucleotides, and a 3' hydroxyl terminus; a second probe which is a PNA-DNA chimera or an oligonucleotide; and a ligase enzyme.

34. The kit of claim 33 in which the chimera probe and/or the second probe is non-radioisotopically labelled.

35. The kit of claim 33 further comprising a template nucleic acid comprising a sequence complementary to the chimeric probe or containing one or more mismatches relative to the chimeric probe.

36. The kit of claim 33 further comprising a polynucleotide kinase.

37. A duplex hybrid comprising:
a PNA-DNA chimeric probe, a second probe which is a PNA-DNA chimera or an oligonucleotide, and a template nucleic acid with a sequence complementary to the chimeric probe or containing one or more mismatches relative to the chimeric probe,

wherein a terminus of the second probe hybridizes adjacent to a terminus of the chimeric probe on the template and said termini can be ligated together by a ligase enzyme.

38. The duplex hybrid of claim 37 in which the chimeric probe and/or the second probe is non-radioisotopically labelled.

39. The duplex hybrid of claim 37 further comprising a polynucleotide kinase.

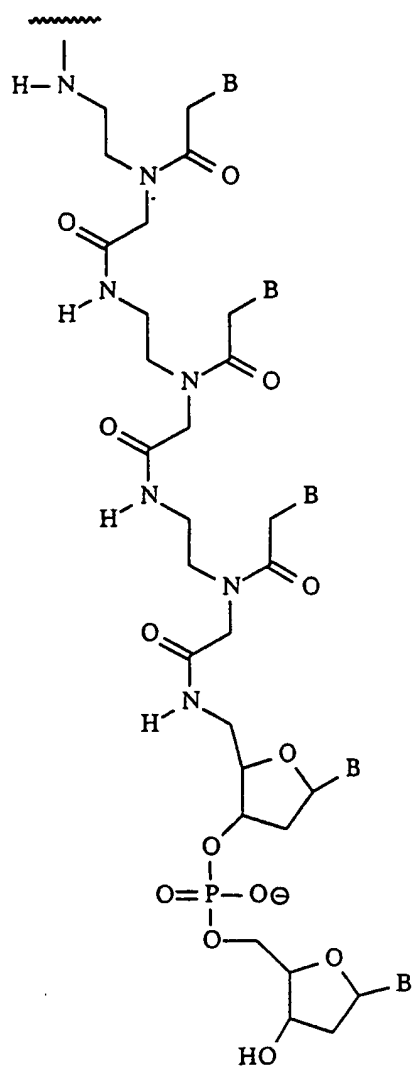


Figure 1A

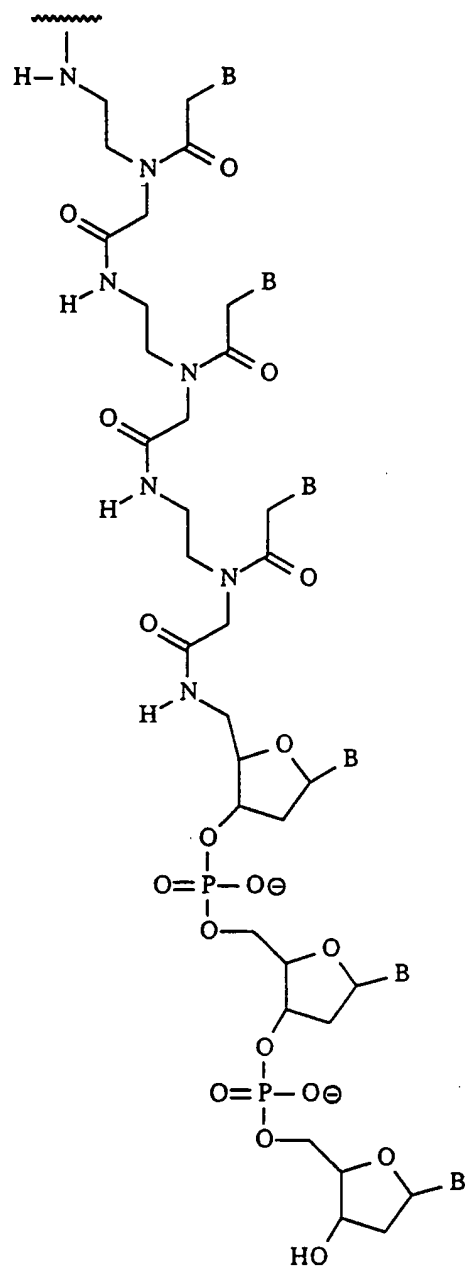
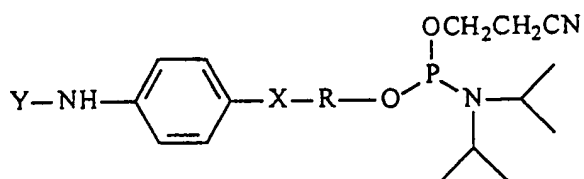
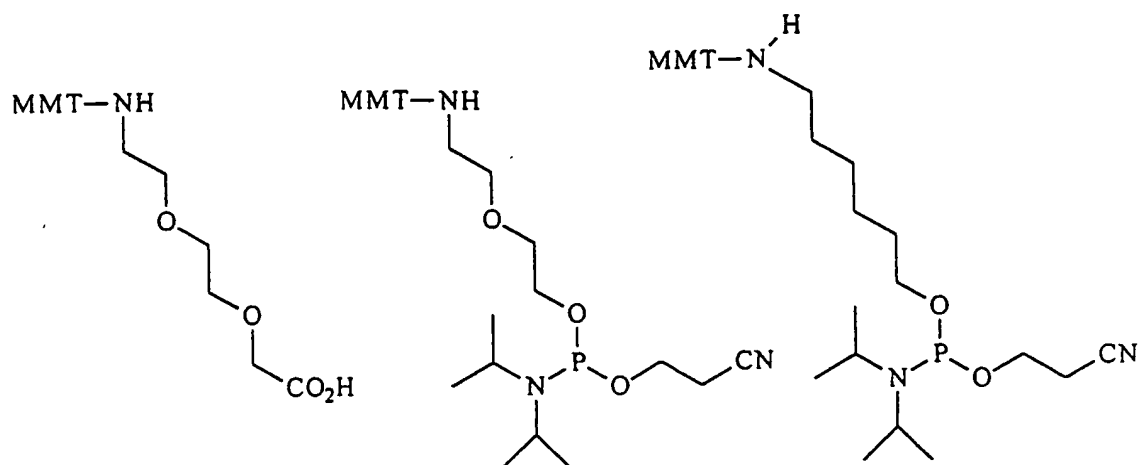


Figure 1B

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X = amide, urea, CH₂, O, or R

R = (CH₂)_n or (CH₂CH₂O)_n
n = 1 - 10

Y = trifluoroacetyl, Fmoc, trityl, MMT, DMT

Figure 2A

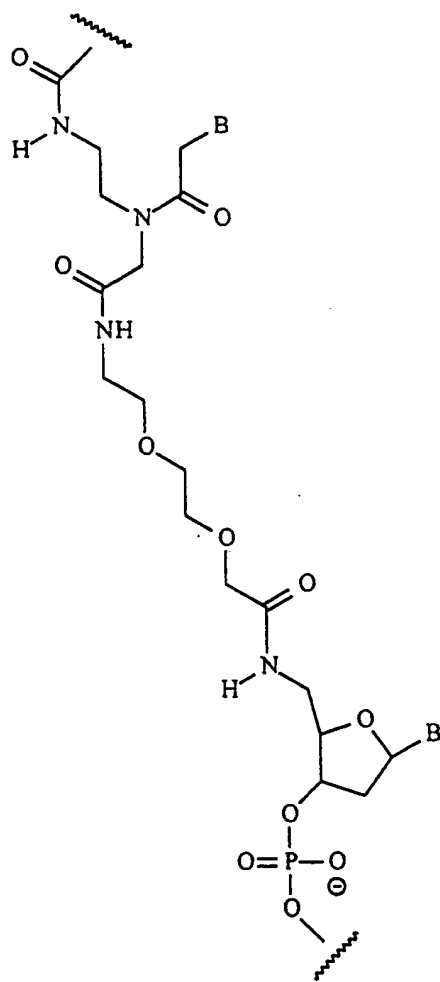


Figure 2B

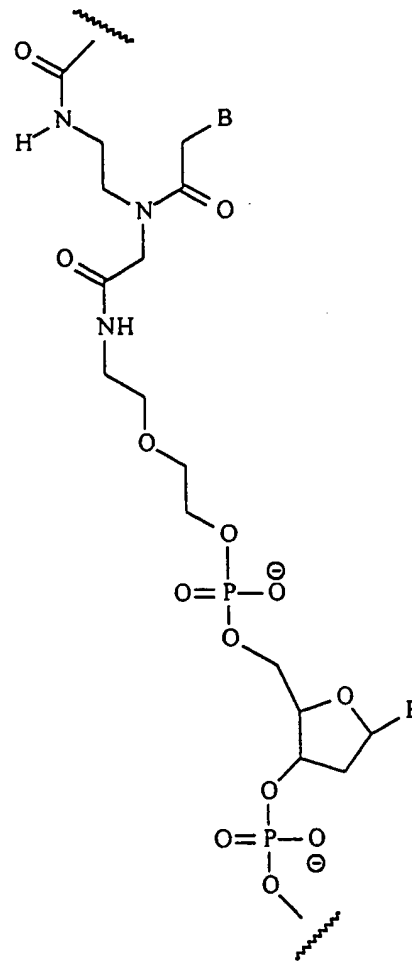


Figure 2C

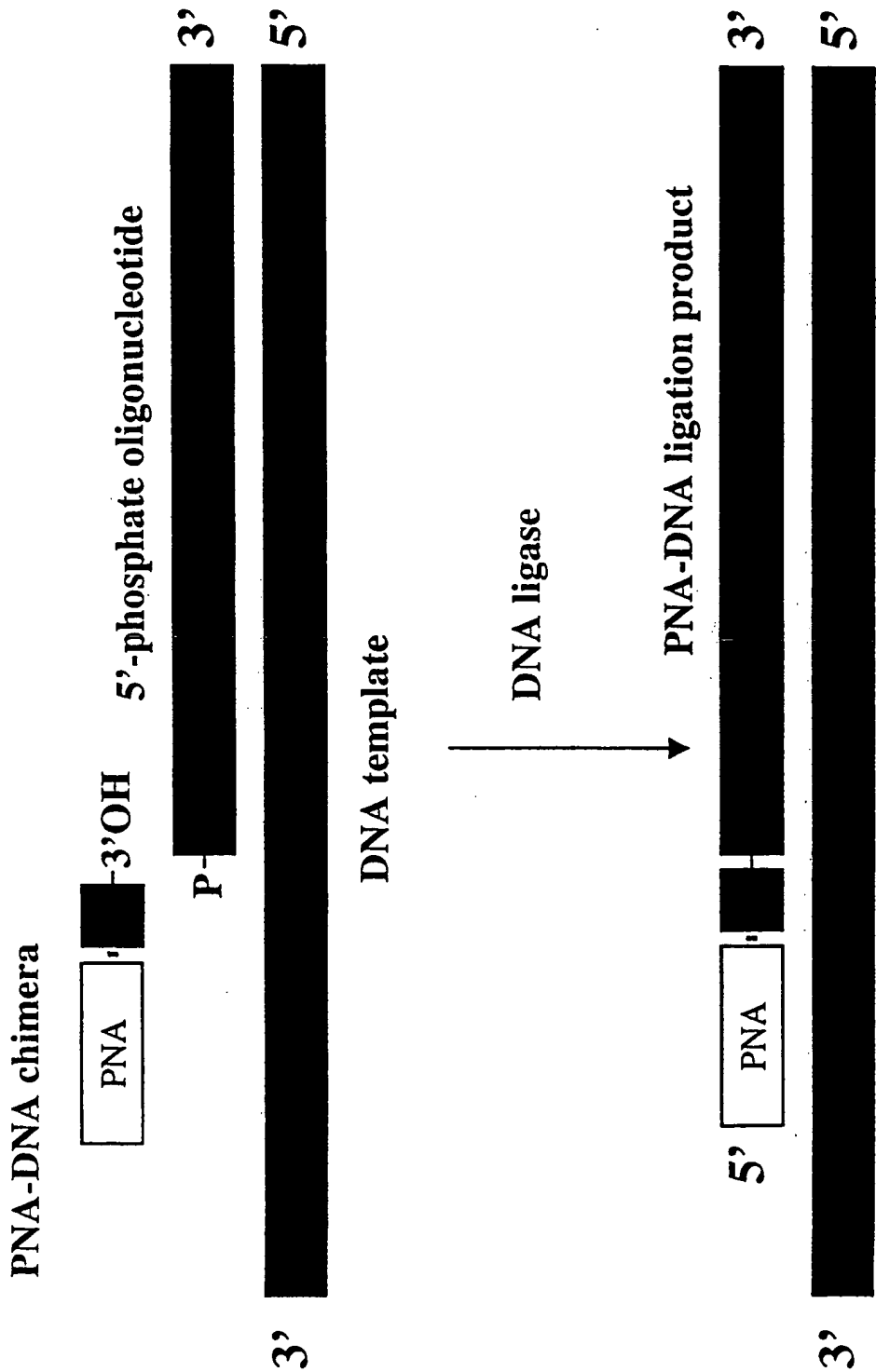
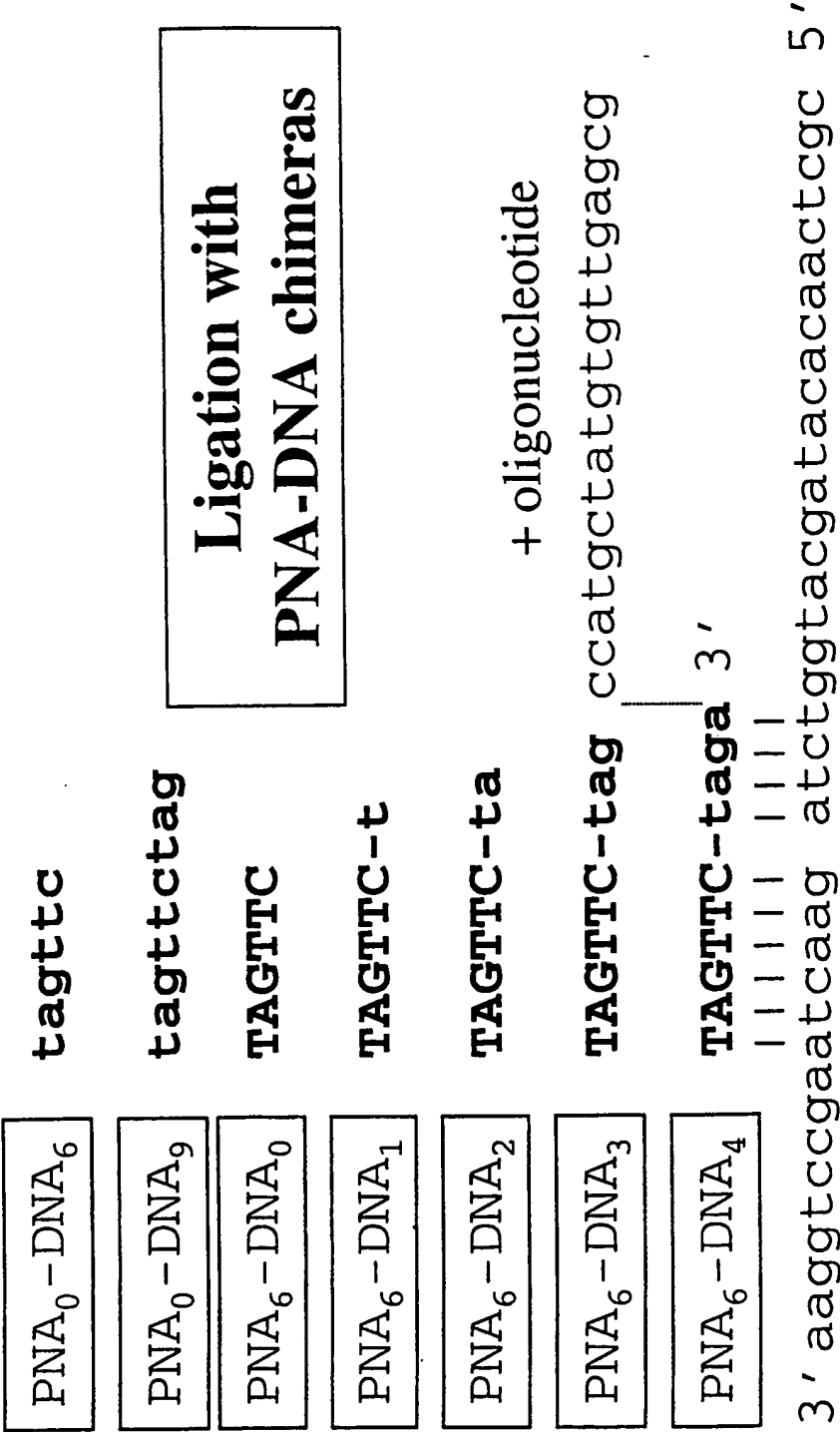


Figure 3A



Perfect match DNA template, 38nt
UPPER CASE - PNA, lower case - DNA

Figure 3B

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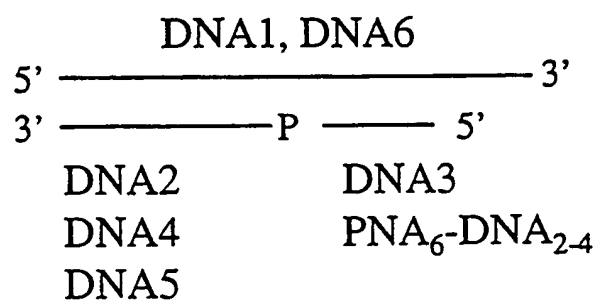
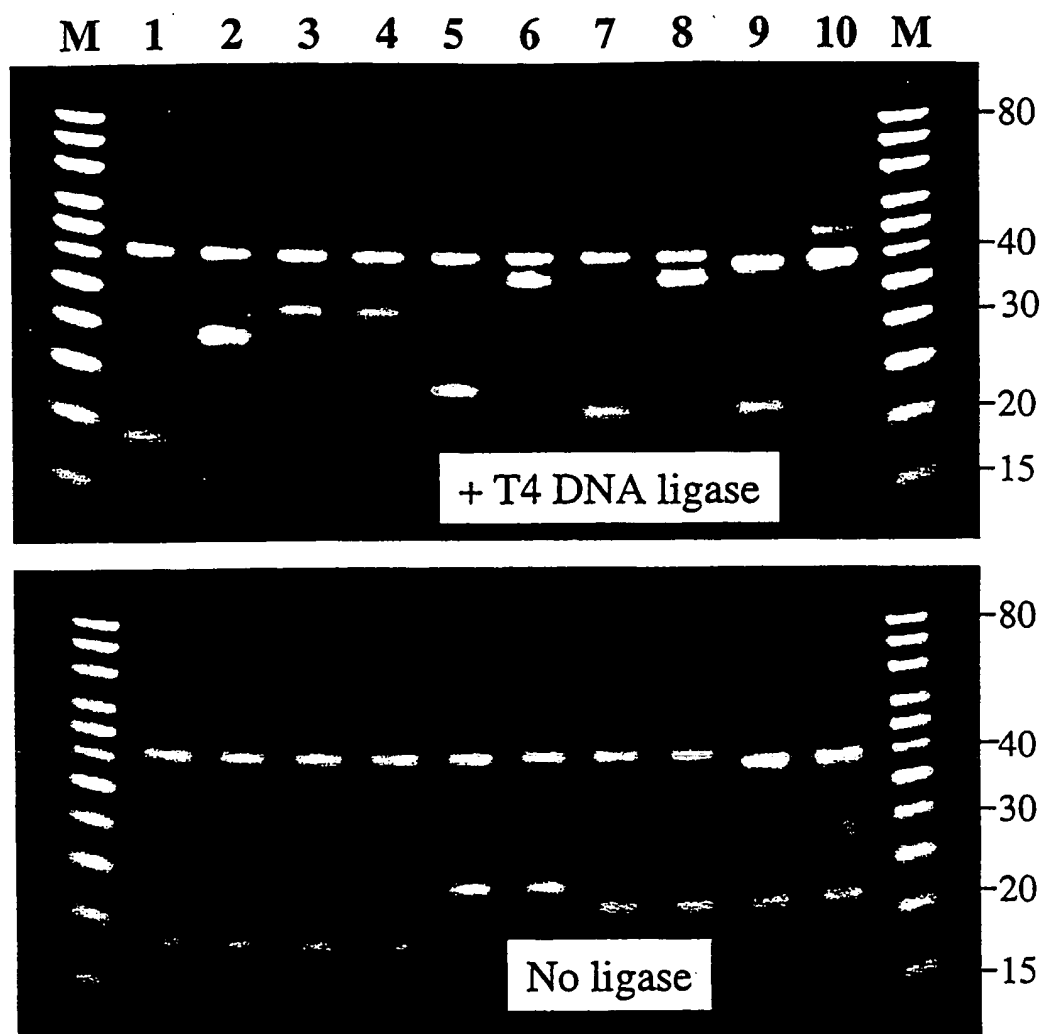
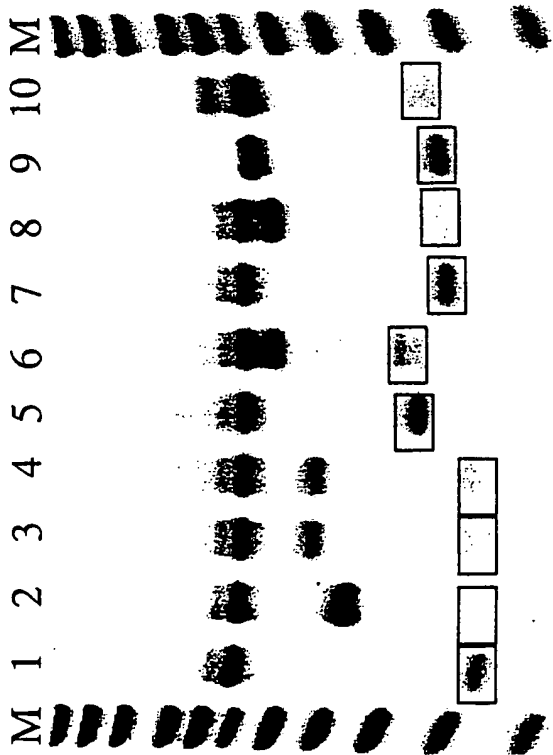


Figure 4A

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Quantitative estimate of PNA-DNA ligation



SpotDenso Results						
Black is 0; White is 255						
#	IDV	%	AREA	AVG	BACK	
1n	21728	12.8	709	31	69	
2n	5517	3.3	709	8	68	
3n	11377	6.7	709	16	69	
4n	11392	6.7	709	16	69	
5n	30085	17.7	709	42	70	
6n	15565	9.2	709	22	70	
7n	24841	14.6	709	35	69	
8n	9719	5.7	709	14	68	
9n	26531	15.6	709	37	69	
10n	12918	7.6	709	18	67	
IDV=Integrated Density Value						
* based on Integrated Density Value						

Figure 4B

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Ligation with T4 DNA ligase

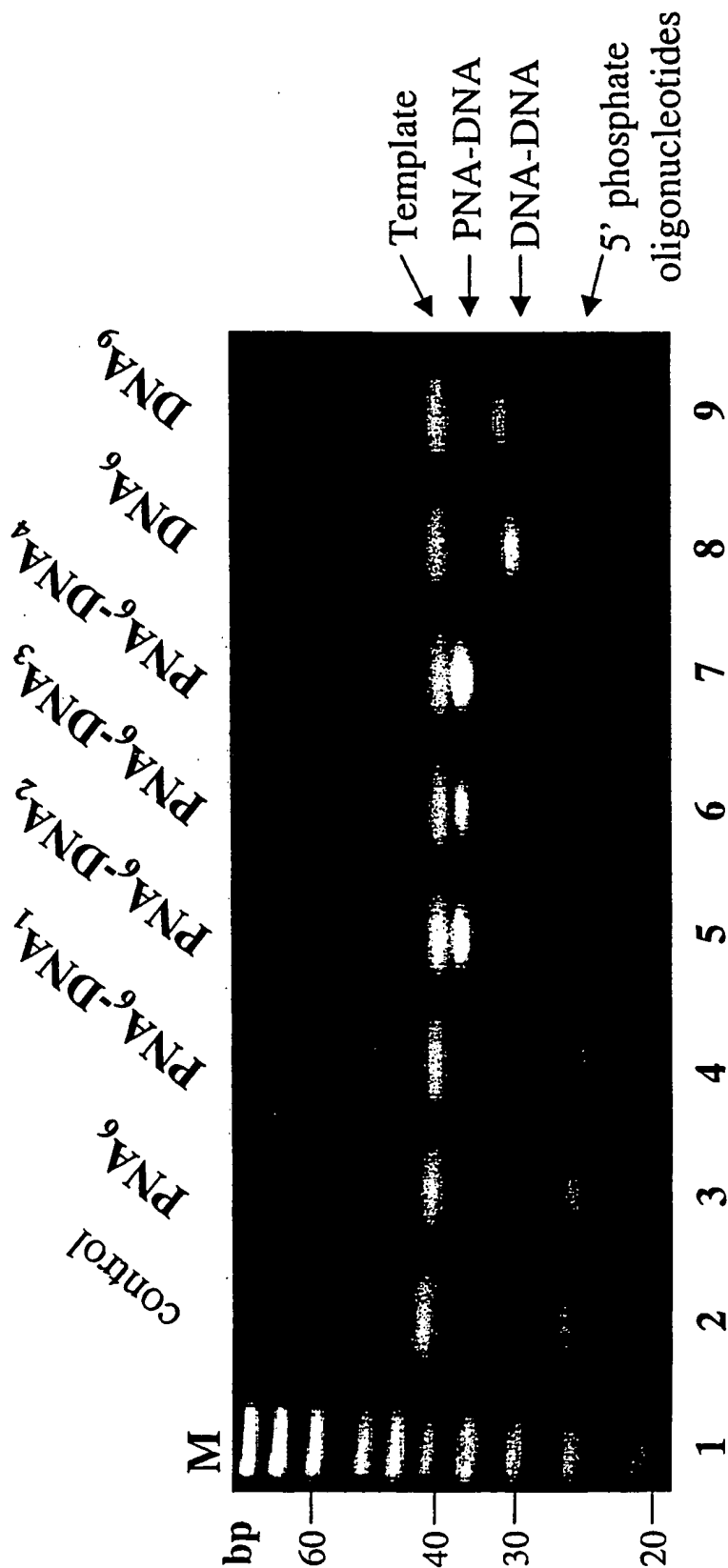


Figure 5A

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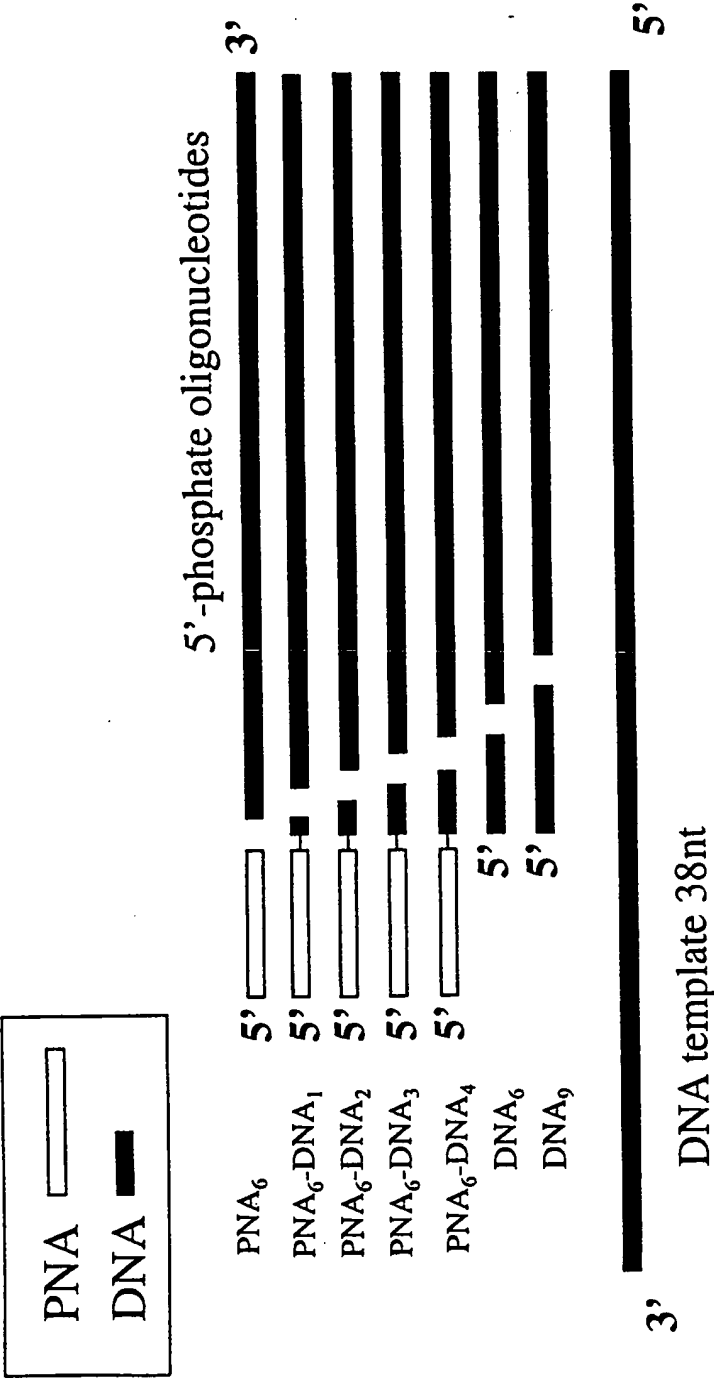


Figure 5B

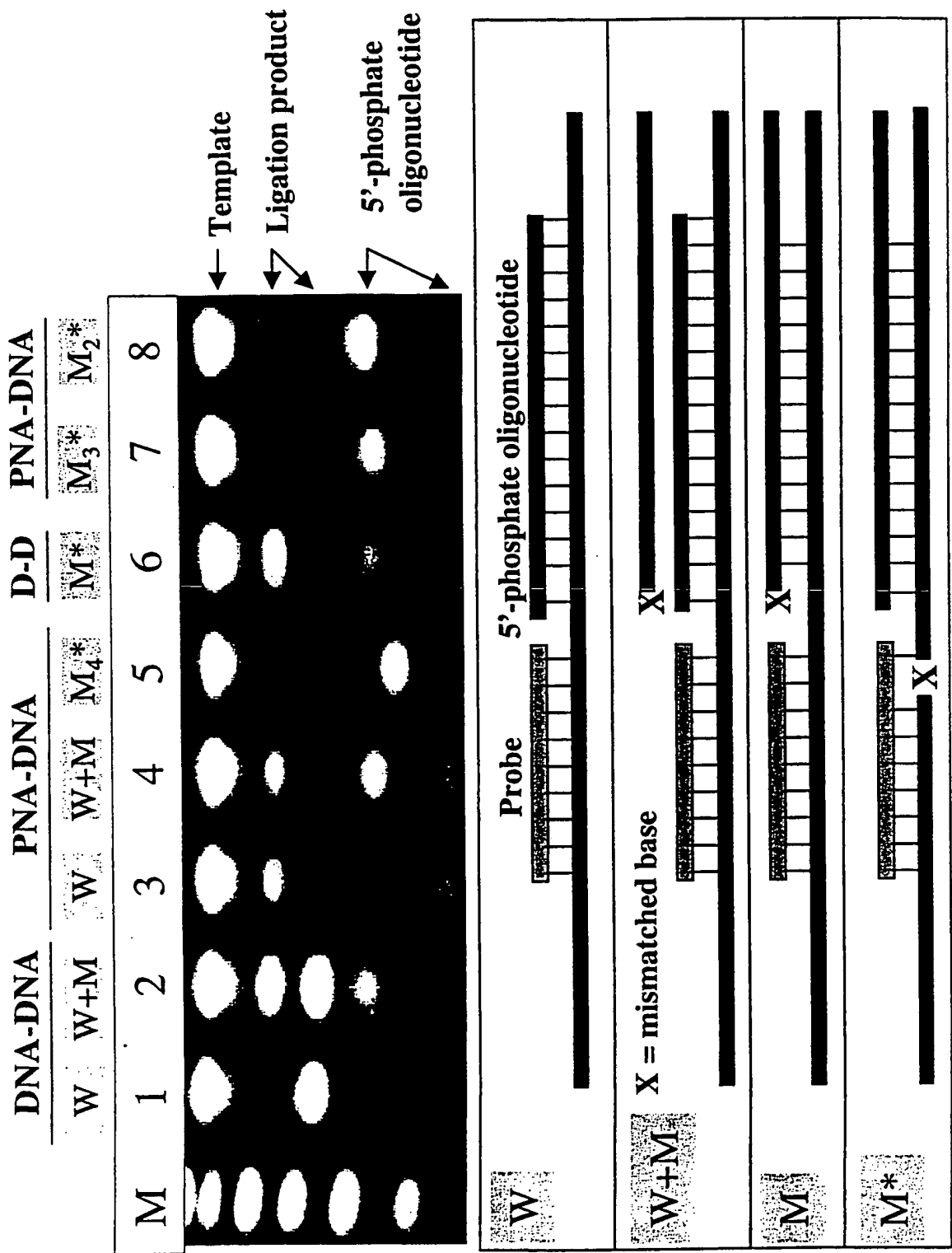


Fig. 6

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MALDI-TOF MS of ligation reaction without ligase

PerSeptive Biosystems/Vestec Products
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 This File # 6 = C:\WINDOWS\DESKTOP\DATA\MALDI--1990216--199021632.MS
 Comment: PNA-DNA ligation no 14

Method: DNAPR
 Accelerating Voltage: 25000
 Sample: 378
 Negative Ions: OFF
 Laser: 2200
 Scans Averaged: 192
 Pressure: 2.42e-07
 Low Mass Gate: 2000.0
 Collected: 2/17/99 4:20 PM

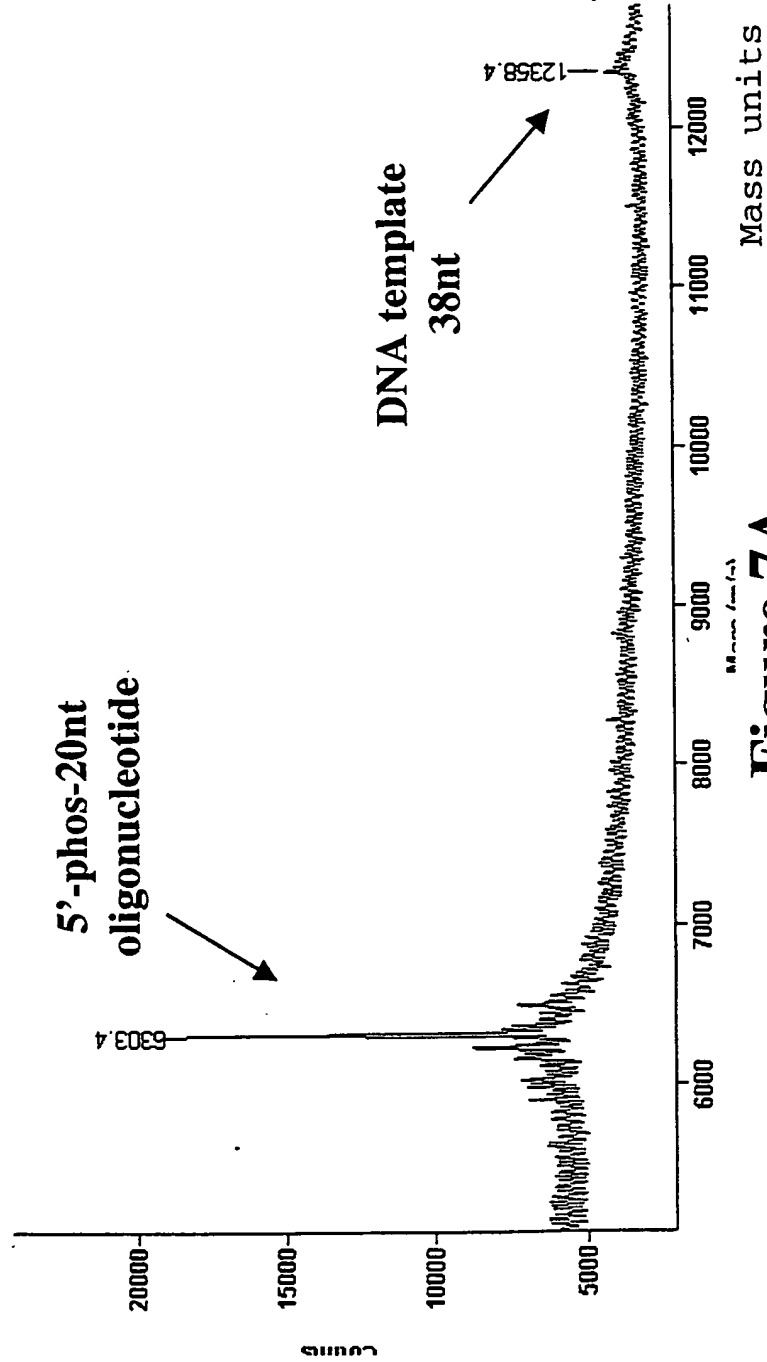


Figure 7A

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MALDI-TOF MS of PNA-DNA ligation product



PerSeptive Biosystems/Vestec Products

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This File # 2 : C:\WINDOWS\DESKTOP\DATA\MALDI--1990216--1\SMOOTH.MS

Comment: PNA-DNA ligation w T4

Method: DNAPR Laser: 2200
Accelerating Voltage: 25000 Scans Averaged: 46
Sample: 378 Pressure: 2.46e-07
Negative Ions: OFF Low Mass Gate: 2000.0
Collected: 2/17/99 4:18 PM
Savitsky-Golay Order = 2 Points = 10

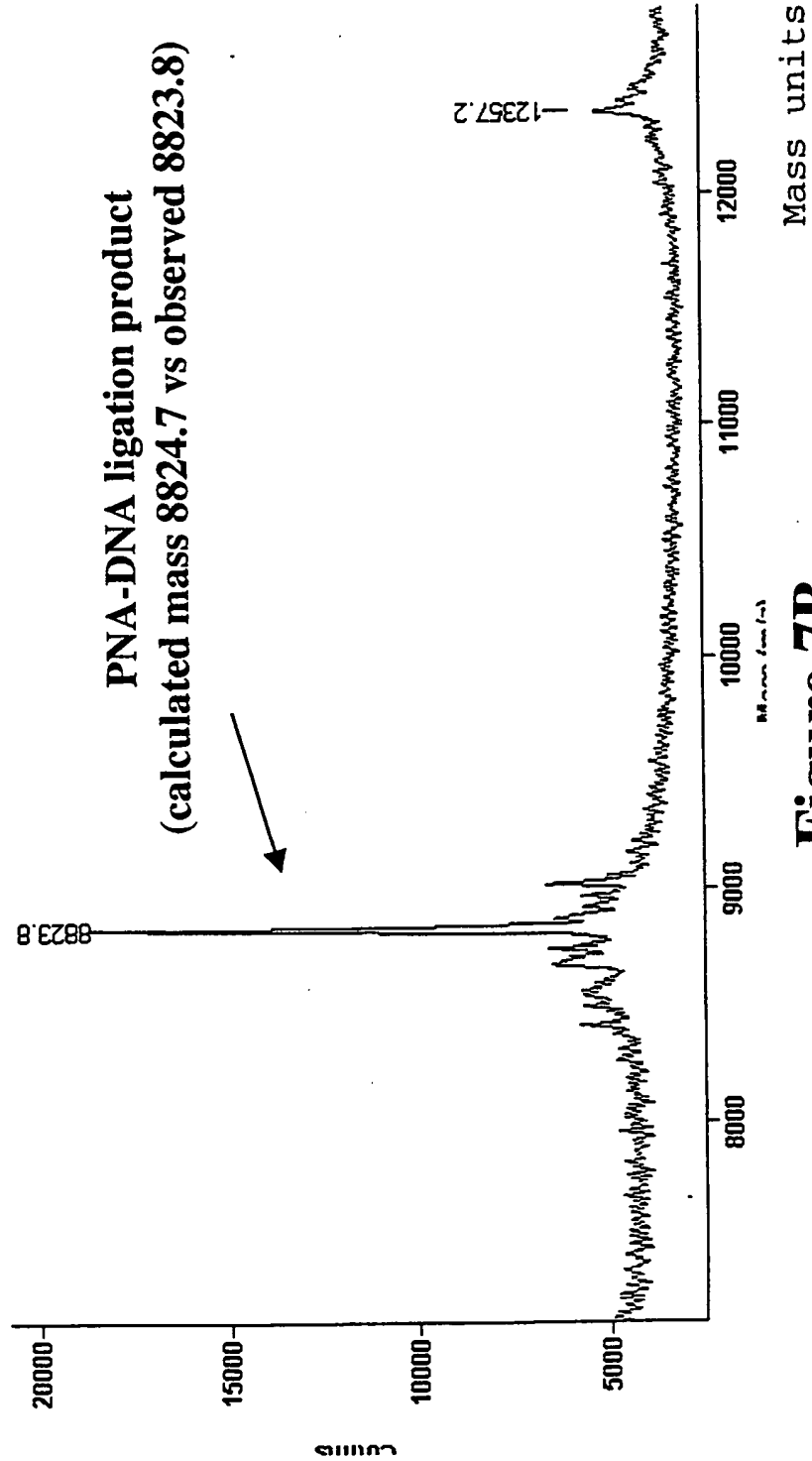


Figure 7B

Multiplex OLA by PNA-DNA chimera ligation

Mismatch base at 5'-phosphate terminus of oligonucleotides of different lengths or mobility modifiers

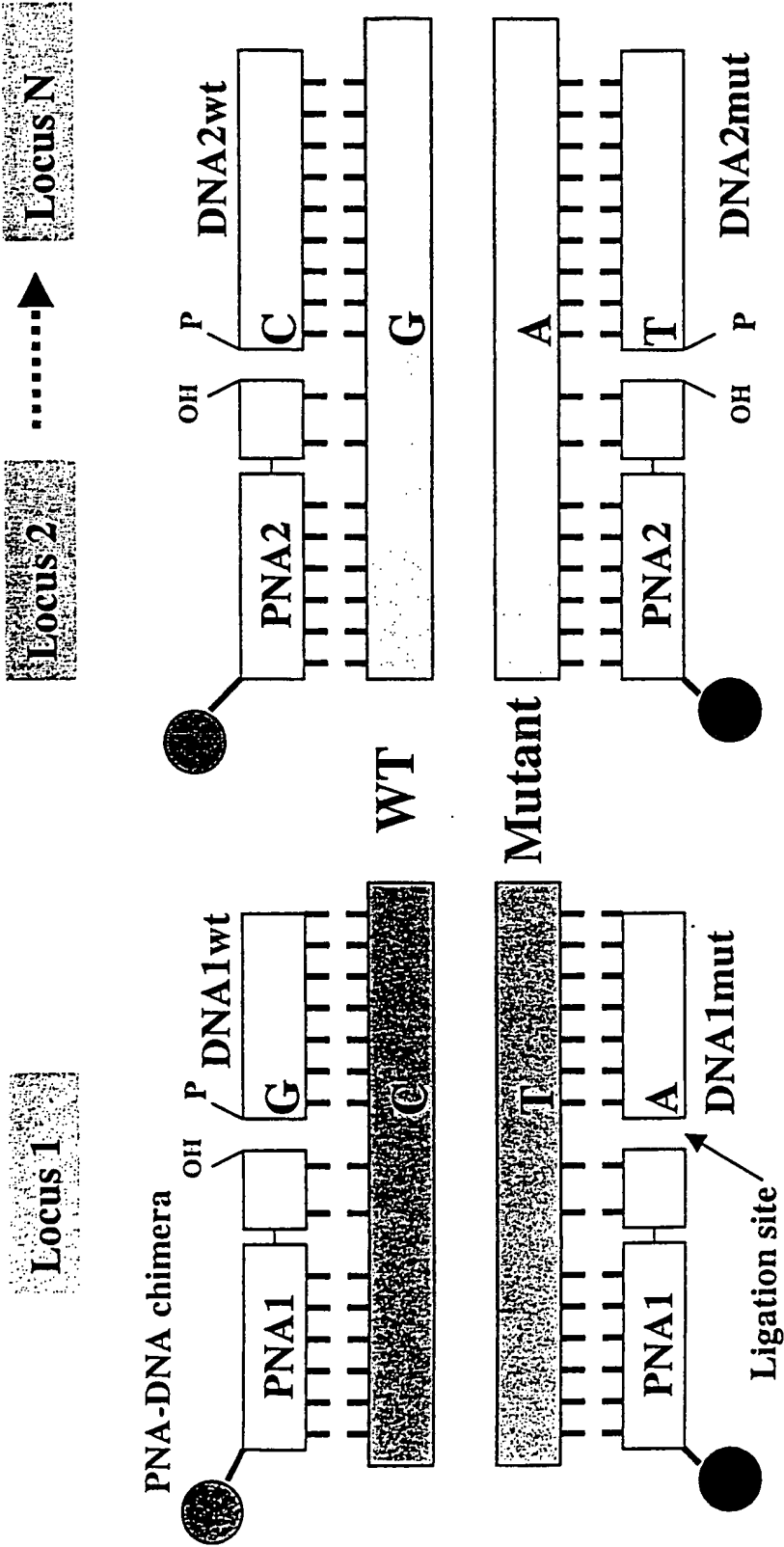


Figure 8B

Multiplex OLA by PNA-DNA chimera ligation

Mismatch base at 3'-terminus of PNA-DNA chimera of different lengths or mobility modifiers

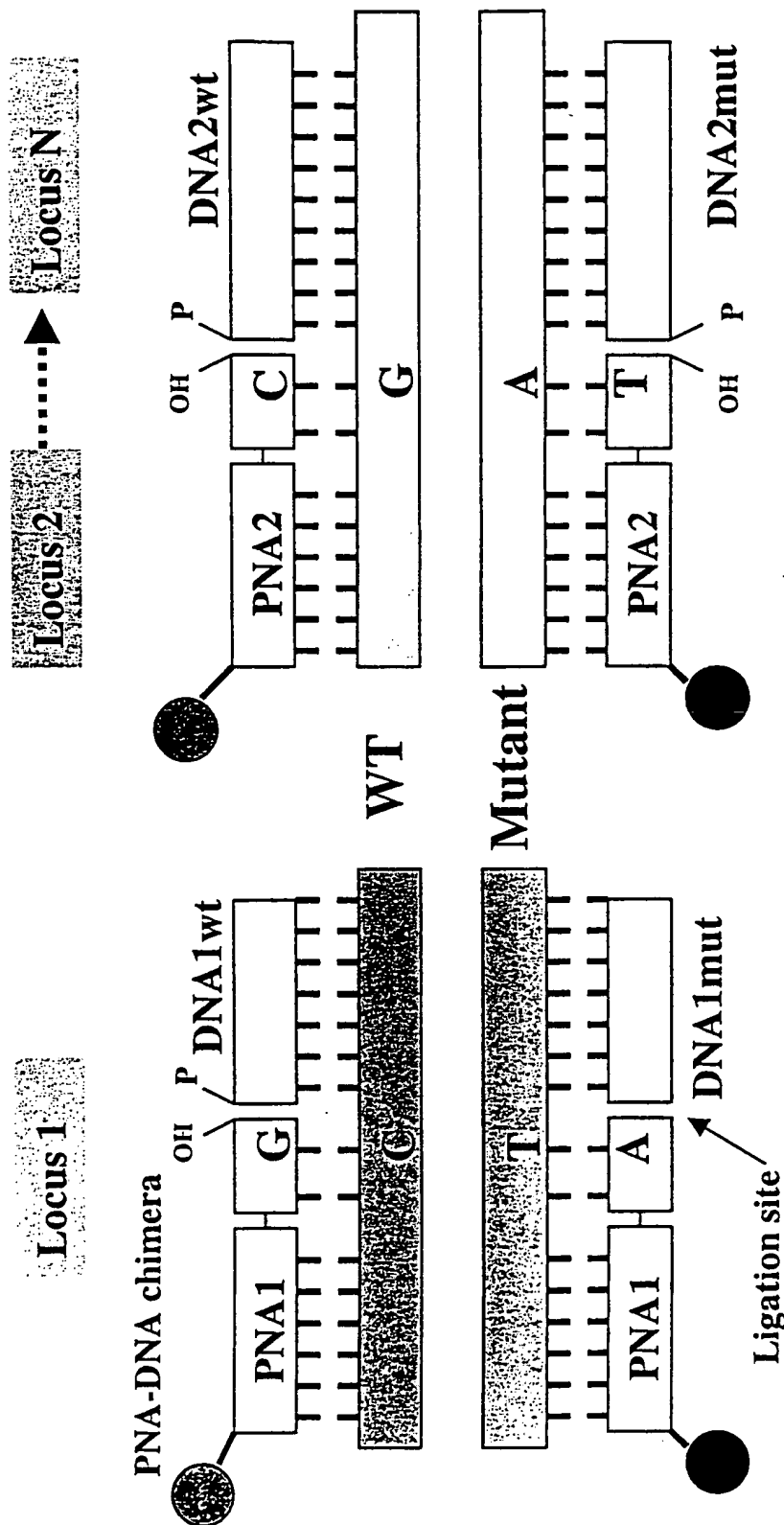


Figure 8C

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PNA OLA for human pCFTR621G-T: Exon 4

Wild-type

5'-phosphate, 3'-HEX oligonucleotide

PNA-DNA chimera

cct tct tat a-HEX 3'

5' C AAG GAA GTA tta

3'cg gac acg ttc ctt cat aat gga aga ata ttt agt ttg att 5'

Mutant (C to T)

act tct tat a-HEX 3'

5' C AAG GAA GTA tta

3'cg gac acg ttc ctt cat aat tga aga ata ttt agt ttg att 5'

Figure 9A

PNA OLA for human pCFTR1078delT: Exon 7

Wild-type

5'-phosphate, 3'-TET oligonucleotide

PNA-DNA chimera 5't ttg tgg tgt ttt t-TET 3'

5' CT TCT CAG GGT tc

3'ga aga aga gtc cca aga aac acc aca aaa ata gac acg 5'

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Mutant (T deletion)

5' ttg tgg tgt ttt t-TET 3'

5' CT TCT CAG GGT tc

3'ga aga aga gtc cca ag- aac acc aca aaa ata gac acg 5'

Figure 9B

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PNA OLA for human pCFTRG551D: Exon 11

Wild-type

5'-phosphate, 3'-FAM oligonucleotide

PNA-DNA chimera

c ctc cac tca gtg tga t-FAM 3'

5' TC TTG CTC GTt ga

3'c ttt aag aac gag caa ctg gag gtg agt cac act aag 5'

Mutant (C to T)

t ctc cac tca gtg tga t-FAM 3'

5' TC TTG CTC GTt ga

3'c ttt aag aac gag caa cta gag gtg agt cac act aag 5'

Figure 9C

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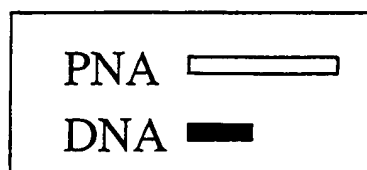
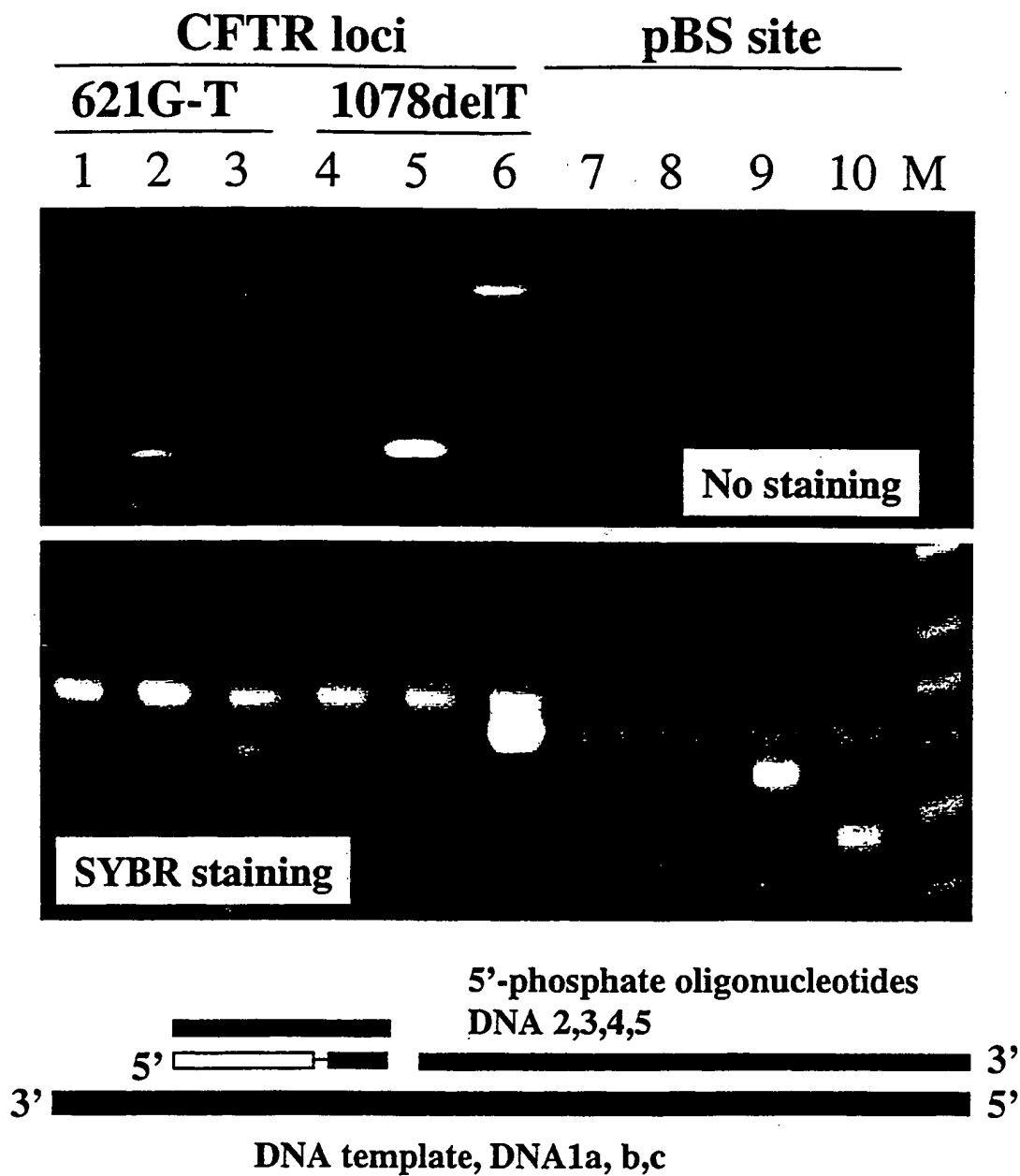


Figure 9D

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EGHOLM, Michael
CHEN, Caifu

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PROBES

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<141> 2000-10-06

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(74) Agents: **ANDRUS, Alex et al.**; Applied Biosystems, 850
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(54) Title: **TEMPLATE-DEPENDENT LIGATION WITH PNA-DNA CHIMERIC PROBES**

(57) Abstract: The invention provides methods, kits, and compositions for ligation of PNA-DNA chimeric probes and oligonu-
cleotides when they are hybridized adjacently to template nucleic acids using ligases and ligation reagents. Structural requirements
of the chimeras for ligation include 5 to 15 contiguous PNA monomer units, 2 or more contiguous nucleotides, and a 3' hydroxyl
or 5' hydroxyl terminus. The chimera and/or oligonucleotide may be labelled with fluorescent dyes or other labels. The methods
include, for example, oligonucleotide-ligation assays (OLA) and single nucleotide polymorphism detection.



WO 01/27326 A3

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C07K14/00

According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, MEDLINE, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>UHLMANN E: "PEPTIDE NUCLEIC ACIDS (PNA) AND PNA-DNA CHIMERAS: FROM HIGH BINDINGAFFINITY TOWARDS BIOLOGICAL FUNCTION"</p> <p>BIOLOGICAL CHEMISTRY HOPPE-SEYLER, WALTER DE GRUYTER, BERLIN, DE, vol. 379, August 1998 (1998-08), pages 1045-1052, XP000884726 ISSN: 0177-3593 cited in the application page 1046 -page 1047, left-hand column page 1049 figure 6</p> <p style="text-align: center;">--- -/--</p>	1-39

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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Name and mailing address of the ISA

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van Klompenburg, W

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/27730

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KOZLOV IGOR A ET AL: "A method for the 32P labeling of peptides or peptide nucleic acid oligomers." BIOCONJUGATE CHEMISTRY, vol. 9, no. 4, July 1998 (1998-07), pages 415-417, XP002190025 ISSN: 1043-1802 abstract; figure 1 ----	37-39
A	EP 0 829 542 A (HOECHST AG) 18 March 1998 (1998-03-18) page 2 -page 4 ----	1-39
A	WO 99 49293 A (BOSTON PROBES INC.) 30 September 1999 (1999-09-30) page 14 -page 15, line 24 page 16 -page 17, line 2 examples 10-12; table 1 ----	12-23
A	WO 96 31622 A (ISIS INNOVATION ; SOUTHERN EDWIN MELLOR (GB); PRITCHARD CLARE ELIZA) 10 October 1996 (1996-10-10) claims 1-13; figures 3,4,6; examples 1,2,5-8 -----	1-39

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/27730

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